REMARKS

In response to the Office Action of January 29, 2003, Applicants have amended the claims, which when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested. In the Office Action of January 29, 2003, the Examiner has made final the previously-issued restriction requirement. Thus, claims 1, 4-10, and 13-23 are under consideration and claims 2, 3, 11, 12, 24-34, and 416 are withdrawn from consideration. Claims 47-50 are newly added and find support in the specification as originally filed. For example, support for newly-added claims 47 may be found throughout the specification, e.g. on page 21, lines 16-26, page 23, lines 9-10, and page 63, lines 6-9. Support for newly added Claim 48 may be found throughout the specification, e.g., page 21, lines 16-26. Support for newly added claim 49 may be found throughout the specification, e.g., page 22, final paragraph, lines 1-3 under "Detecting and mapping of related polynucleotide sequences". The subject matter recited in newly-added claim 50 finds support throughout the specification, e.g., page 34, lines 5-8 and page 33, final paragraph.

Claim 1 has been objected to because it allegedly recites sequences of non-elected inventions. Claim 1 has been amended so that non-elected sequences are no longer recited. Claim 4 has been objected to because it allegedly depends from claims directed to non-elected inventions. Claim 4 has been canceled without prejudice. Withdrawal of the objection to claims 1 and 4 is therefore warranted.

The specification has been objected to because an abstract of the disclosure has not been provided as required by 37 C.F.R. 1.72(b). Submitted herewith on a separate sheet is an abstract

of the disclosure. Withdrawal of the objection to the specification is therefore respectfully requested.

Claims 1, 4, 5, 6-10, and 13-23 have been rejected under 35 U.S.C.§ 112, first paragraph, as allegedly violative of the written description requirement of 35 U.S.C. § 112, first paragraph. Under the Written Description Guidelines, *Fed. Reg. Vol. 66,* No. 4, January 5, 2001, pages 1099-1111, the Examiner has the initial burden, after a thorough reading and evaluation of the application, of presenting evidence or reasons why one skilled in the art would not recognize that the written description of the invention provides support for the claims. The Examiner has stated that "[t]he specification does not describe or characterize any nucleotide sequence hybridizing with the complementary strand of SEQ ID NO:7 or a nucleic acid encoding SEQ ID NO:8 under stringent hybridization conditions, any nucleotide sequence encoding a protein having an amino acid sequence at least 60% identical to the amino acid sequence of SEQ ID NO:8, any nucleotide therefore protein encoded by the nucleotide sequence of (da) to (dd), or any nucleic acid molecule at least 15 nucleotides in length hybridizing specifically with a DNA sequence of claim 1." Office Action, page 4, line 18 to page 5, line 2.

In response to the rejection of Claims 1, 4-10, and 13-23, Applicants respectfully submit the following. A showing that the written description of an invention is sufficient to inform a skilled artisan at the time the application was first filed, that applicant was in possession of the claimed invention, may be made by many different ways. One way to show possession of the invention is by actual reduction to practice.

Reduction to practice is only *one* way to show possession of the invention. Thus, with respect to other species of DNA sequences encoding a cell cycle interacting protein encompassed by the rejected claims, Applicants may use other indicia to show possession of the invention.

Thus, the written description requirement for a claimed genus may be satisfied by disclosure of "relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus." Written Description Guidelines, *Fed. Reg. 66*(4), page 1106.

As presently amended, the rejected claims recite in relevant part:"[a]n isolated DNA molecule encoding a cell cycle interacting protein or encoding an immunologically active and/or functional fragment thereof, wherein said cell interacting protein binds to a cyclin dependent kinase (CDK) having a PPTLARE cyclin binding motif" Support for the amended claim language may be found throughout the specification. To illustrate, Example 4, lines 4-5 discloses that "[u]nexpectedly, the Vb89 clone interacts with CDC2bAt, but not with CDC2aAt in the two hybrid system. The interaction of Vb89 with CDC2bAt highlights an important role of Vb89 in cell cycle control." Page 4, lines 17-26 discloses:

> In Arabidopsis, thusfar only two CDK genes have been characterized in detail *CDC2aAt* and *CDC2bAt*, of which the gene products share 56% amino acid identity. Both CDKs are distinguished by several features. First, only *CDC2aAt* is able to complement yeast $p34^{CDC2/CDC28}$ mutants. Second, CDC2aAt and CDC2bAt bear different cyclin-binding motifs (PSTAIRE and PPTALRE, respectively), suggesting they may bind distinct types of cyclins. Third, although both *CDC2aAt* and *CDC2bAt* show the same spatial expression pattern, they exhibit a different cell cycle phase-specific regulation. The *CDC2aAt* gene is expressed constitutively throughout the whole cell cycle. In contrast, *CDC2bAt* mRNA levels oscillate, being most abundant during the S and G_2 phases.

Thus, the claims as amended are fully supported by the written description as indicated in the written description guidelines, since the disclosure describes the relevant identifying characteristics recited in the claims (binding to a cyclin dependent kinase having a PPTLARE

cyclin binding motif), i.e., functional characteristics coupled with a known or disclosed correlation between function and structure sufficient to show applicant was in possession of the claimed genus." *See Fed. Reg.* 66(4):1106.

The proper test for sufficiency of description in a patent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter. *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983). Exactly how the specification allows one skilled in the art to recognize that an applicant had possession of the claimed invention is not material. *In re Smith,* 481 F.2d 910, 178 USPQ 279 (CCPA 1973). Typically, an applicant conveys that he or she is in possession of the invention by use of descriptive means such as "words, structures, figures, diagrams, formulas, etc., that set forth the claimed invention." *Lockwood v. American Airlines,* 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). To comply with the description requirement, it is not necessary that the application describe the invention *ipsis verbis*. *In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971). What is required is that an ordinarily skilled artisan recognize from the disclosure that applicants invented the subject matter of the claims, including the limitations recited therein. *In re Smith*, 481 F.2d at 915, 178 USPQ at 284.

It is respectfully submitted that the claims as presently amended are sufficiently supported by the written description provided in the specification since, based on the foregoing remarks, one skilled in the art would reasonably believe that Applicants invented the subject matter recited therein. Withdrawal of the rejection of Claims 1, 4-10 and 13-23 under 35 U.S.C. §112, first paragraph, is therefore respectfully requested.

Claims 1, 4, 9, 10, 18, 19, and 22, and claims 5-10, 13-17, 20-21 and 23 dependent thereon, have been rejected under 35 U.S.C. 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 1, 4, 10 and 19 are specifically objected to for the recitation of "cell cycle interacting protein" since according to the Examiner, "it is unclear what function the protein exhibits, as the nature of the specific interaction is not specified, and the relationship between the protein and the cell cycle is unclear." As presently amended, Claim 1 recites in relevant part: "wherein said cell cycle interacting protein binds to a cyclin dependent kinase (CDK) having a PPTALRE cyclin binding motif." Claim 4 is presently canceled without prejudice. Claims 10 and 19 depend from presently amended claim 1.

The Examiner has also objected to the recitation in claim 1 of "VB89." As presently amended, Claim 1 no longer recites "VB89. The recitation of "stringent hybridization conditions" in Claim 1 is also objected to by the Examiner as it is allegedly unclear what constitutes such conditions. As presently amended, Claim 1 recites stringent hybridization conditions well known to those of skill in the art as well as taught by the present application. The stringent hybridization conditions presently recited in Claim 1, i.e., hybridization in 4X SSC at 65° C, followed by washing in 0.1X SSC at 65° C, or hybridization in 50% formamide, 4X SSC at 42° C, followed by washing in 0.1X SSC, is specifically disclosed in the present application at page 13, lines 7-10. The recitation of "CDK" in Claim 1 is also objected to by the Examiner. As presently amended, the preamble of Claim 1 recites "cyclin dependent kinase" followed by the well known acronym "CKD" in parentheses.

Claim 18 has been rejected under the second paragraph of 35 U.S.C. §112, for reciting "enhanced". As presently amended, Claim 18 recites in relevant part: "in which plant cell

division and/or growth is enhanced *compared to the corresponding wild type plant.*" Claim 18 is also rejected for reciting "environmental stress." According to the Examiner, it is unclear what types of stress are encompassed by the claim, as any aspect of the environment can impose stress on a plant. Applicants traverse the rejection of Claim 18 for reciting "environmental stress" and respectfully submit that it is well known among those skilled in the art that plants mainly suffer from a limited number of abiotic stresses: salt, nutrition deprivation, drought, heat, freezing, chilling and to a lesser extent anaerobia and light. *See e.g.,* Chapter 26: Stress Physiology in *Plant Physiology*, Salisbury and Ross, 1992, Wadsworth Inc., Ca, USA, pp. 575-600, submitted herewith as Exhibit A. Basis for use of the term "environmental stress" may also be found in the present application, e.g., pp. 34, lines 5-8 where it is disclosed "[f]urthermore, overproduction of the cell cycle interacting protein of the invention enhances growth and results in cell division to be less sensitive to an arrest caused by environmental stress such as salt, nutrient deprivation, drought, chilling, and the like." Page 37, lines 8-9 also refer to certain environmental conditions such as pathogens, anaerobia, and light. Finally, Claim 22 has been rejected due to the recitation of "deficiency" since it is allegedly a relative term lacking a comparative basis. As presently amended, Claim 22 recites in relevant part: "a deficiency in plant cell division and/or growth compared to the corresponding wild type plant."

In view of the amendments to the claims and the remarks hereinabove, withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, of Claims 1, 4, 9, 10, 18, 19 and 22, and Claims 5-10, 13-17, 20-21 and 23 dependent thereon, is respectfully requested.

Claims 1 and 4-5 have been rejected under 35 U.S.C. §101 as allegedly directed to non-statutory subject matter. As presently amended, Claim 1 recites in relevant part: "[a]n isolated DNA molecule." Claim 4 is canceled without prejudice. Claim 5 depends from Claim 1, and

thus also recites "an isolated DNA molecule." Withdrawal of the rejection of Claims 1 and 4-5 is therefore warranted.

Claims 1, 4-10, and 13-23 have been rejected under 35 U.S.C. § 101 as allegedly not supported by either a specific and substantial asserted utility or a well established utility. The same claims have also been rejected under 35 U.S.C. § 112, first paragraph, as allegedly violative of the how-to-use provision. The Examiner has based the rejection at least in part, on the fact that no specific function has been demonstrated for the polypeptide encoded by the claimed DNA sequence. In response to the rejection, Applicants direct the Examiner to page 72 of the application where the presently claimed invention with respect to the gene and corresponding protein of SEQ ID NOs: 7 and 8 are described. As described therein, the presently claimed DNA sequence has overall perfect homology with a partial cDNA Hal3, isolated from *Arabidopsis thaliana* and entered on the gene databank.

As described on page 72 of the present application, the *HAL3* gene was first isolated as a halotolerant gene from *Saccharomyces cerevisiae*, encoding a protein which regulates the cell cycle and tolerance to salt stress through inhibition of the PPZ1 type-1 protein phosphatase Based on such homology to the *Hal3* gene of *S. cerevisiae,* the inventors of the present application teach that the presently claimed DNA sequence is *inter alia*, useful for enhancing growth and conferring salt tolerance on plants and/or improved growth under such conditions. *See* specification, page 72, final paragraph.

Indeed, following the teachings of the present invention, Espinosa-Ruiz et al. (1999) *The Plant Journal 20(5)*:529-539, submitted herewith as Exhibit B, disclose the isolation of two *Arabidopsis thaliana* genes which show homology with *S. cerevisiae* HAL3. Transgenic plants overexpressing *AtHAL3a* show a faster growth rate than the wild type, while *AtHAL3a* antisense

plants produce the opposite phenotype. Some plants showed improved salt and osmotic tolerance compared to wild-type and transgenic antisense plants. Further, sense transgenic plants developed roots and true leaves and continued growing under stress conditions, while wild type plants mainly remained at the cotyledon stage. *See* Espinosa-Ruiz et al. (1999) page 536, column 1, lines 1-17,submitted herewith as Exhibit B.

The protein corresponding to the presently claimed isolated DNA molecules, i.e., SEQ ID NO:8 and 7 respectively, is highly homologous to AtHAL3a (83% overall identity and 87% identity when the N-terminal amino acids before the second methionine are not taken into account) of Espinosa-Ruiz. The protein set forth in SEQ ID NO:8 and AtHAL3a also share the same expression pattern.

When a patent application claiming a nucleic acid asserts a specific, substantial, and credible utility and *bases the assertion upon homology to existing nucleic acids or proteins having an accepted utility, the asserted utility must be accepted by the examiner unless the Office has sufficient evidence or sound scientific reasoning to rebut such an assertion.* A rigorous correlation need not be shown in order to establish practical utility; 'reasonable correlation' is sufficient. Utility Guidelines, *Fed. Reg. Vol. 66*, No. 4, Friday, January 5, 2001, Notices, page 1099, citing *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1565, 39 USPQ2d 1895, 1900 (Fed. Cir. 1996), emphasis added. There is no *per se* rule regarding homology, and each application should be judged on its own merits, taking into account both the nature and degree of homology. Utility Guidelines, page 1099. Only one specific, substantial and credible utility is required to satisfy the statutory requirement. Utility Guidelines, page 1098.

It is respectfully submitted that the present application teaches a specific, and asserted utility as well as a well established utility. This teaching with respect to SEQ ID NOs. 7 and 8 is

based on homology with a halotolerant gene from *S. cerevisiae.* Such a teaching has now been verified by others e.g., Espinosa-Ruiz, using the same homology based approach using the yeast HAL3 protein. "Only where the totality of the record *continues* to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained." Utility Guidelines, page 1099. In addition, since Applicants have now rebutted a rejection based on allegedly lack of utility under section 101, the corresponding rejection imposed under section 112 should also be withdrawn. *Id.* Withdrawal of the rejection of Claims 1, 4-10, and 13-23 under 35 U.S.C. §§101 and 112, first paragraph, is therefore warranted.

Claims 1 and 4-10 have been rejected under 35 U.S.C.§ 102(b) as allegedly anticipated by DeVeylder et al. (1997) *FEBS Lets. 412*: 446-452. DeVeylder et al., teach a DNA sequence encoding Cks1at which protein binds to both Cdc2a and Cdc2bAt. The Examiner agrees that De Veylder et al. do not teach a DNA sequence having the sequence set forth in SEQ ID NO:7 encoding a protein having the amino acid sequence as set forth in SEQ ID NO:8. According to the Examiner however, the DNA sequence taught by DeVeylder et al. would nonetheless hybridize with SEQ ID NO:7 or a nucleic acid molecule at least 15 nucleotides in length *as the hybridization conditions are undefined.* As discussed fully hereinabove, stringent hybridization conditions are now set forth in the claims. One skilled in the art would not reasonably believe that the DNA sequence taught by DeVeylder et al. would hybridize to the presently claimed DNA molecules under the stringent conditions recited therein. Withdrawal of the rejection of Claims 1 and 4-10 under 35 U.S.C. § 102(b) is therefore respectfully requested.

Claims 1, 4-10, and 13-23 have been rejected under 35 U.S.C. § 103 as allegedly unpatentable over Doerner et al. (1996) *Nature 380*:520-523, in view of DeVeylder et al. (1997)

FEBS Letts. 412:446-452. Doerner et al. describe alteration of root growth by overexpressing cyclin1At under control of the cdc2aAt promoter in *Arabidopsis thaliana*. Doerner et al. do not describe an isolated DNA sequence encoding a cell cycle interacting protein as presently claimed. Indeed, the presently claimed HAL3 gene (SEQ ID NO:7) and corresponding protein (SEQ ID NO:8) is distinct from the Cyclin1At taught by Doerner et al.

As discussed above, DeVeylder et al. describe proteins which interact with CDK. One skilled in the art however, would not reasonably believe that the DNA sequence taught by DeVeylder et al. would hybridize to the presently claimed DNA molecules under the stringent conditions recited therein. Thus, the teachings of DeVeylder et al., do not ameliorate the teachings provided by Doerner et al. Absent a teaching or a suggestion for an isolated DNA sequence encoding a cell cycle interacting protein which binds to a CDK having a PPTLARE cyclin binding motif wherein said DNA sequence is as set forth in (aa) through (af) of presently amended Claim 1, Applicants' claimed invention is not obvious. Withdrawal of the rejection of Claims 1, 4-10, and 13-23 under 35 U.S.C.§ 103(a) is therefore respectfully requested.

In view of the foregoing remarks and amendments to the claims, it is firmly believed that the present application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

Ann R. Pokalsky
Reg. No. 34,697
Attorney for Applicants

DILWORTH & BARRESE
333 Earle Ovington Blvd.
Uniondale, NY 11553
(516) 228-8484

Enclosure: Abstract

ABSTRACT

Provided are DNA sequences encoding cell cycle interacting proteins as well as methods for obtaining the same. Furthermore, vectors comprising said DNA sequences are described, wherein the DNA sequences preferably are operatively linked to regulatory elements allowing expression in prokaryotic and/or eukaryotic host cells. In addition, proteins encoded by said DNA sequences, antibodies to said proteins and methods for their production are provided. Also described is a method for controlling or altering growth characteristics of a plant and/or a plant cell comprising introduction and/or expression of one or more cell cycle regulatory proteins functional in a plant or parts thereof and/or one or more DNA sequences encoding such proteins. Also provided are regulatory sequences controlling the expression of the above described cell cycle interacting proteins. Method for the identification of compounds being capable of activating or inhibiting the cell cycle are described as well. Further described are diagnostic compositions comprising the aforementioned DNA sequences, regulatory sequences, proteins, antibodies, inhibitors and activators. Furthermore, transgenic plant cells, plant tissue and plants containing the above-described DNA sequences and vectors are described as well as the use of the aforementioned DNA sequences, vectors, proteins, regulatory sequences, antibodies and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture.

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26

Stress Physiology

An important branch of environmental physiology is concerned with how plants and animals respond to environmental conditions that deviate significantly from those that are optimal for the organism in question—or, in a broader sense, for organisms in general. As a division of physiological ecology, this field, called **stress physiology**, can contribute to our understanding of what limits plant distribution. Most research in the field, however, is concerned with how adverse environmental conditions limit agricultural yields. One of the first challenges encountered is how to define the word *stress*.

26.1 What Is Stress?

In 1972, Jacob Levitt (see Levitt, 1972, 1980) proposed a definition of biological stress derived from physical science. *Physical stress* is any force applied to an object (for example, a steel bar); *strain* is the change in the object's dimensions (for example, bending) caused by the stress. Levitt suggested that **biological stress** is any change in environmental conditions that might reduce or adversely change a plant's growth or development (its normal functions); **biological strain** is the reduced or changed function.

Recall our discussion of limiting factors and the law of tolerance (Section 25.3). When environmental conditions are such that the plant is responding maximally to some factor (is at or close to the optimum part of the curve in Fig. 25-1), it is not being stressed by that factor. Any change in environmental conditions that results in plant response that is less than the optimum might be considered stressful. Of course, such a concept is sometimes easier to discuss in a theoretical way than it is to apply. Consider a plant suddenly subjected to reduced light levels. Because photosynthesis is immediately reduced, the lower light levels would be the *stress* and the diminished photosynthesis the *strain*. Stem elongation would probably be promoted, too; so, were *high* light levels a stress for stem elongation? Probably we would conclude that the promoted stem elongation rates actually constituted the strain because they led to taller stems with less mechanical strength—but that could be an advantage if the leaves were thereby carried above shading competitors into higher light levels. It's all a question of what is "best" or "normal" for a given plant, and the answer to this question can be highly subjective, depending upon circumstances and judgments. Most studies in stress physiology have been concerned with conditions that are much more obviously stressful; for example, conditions that limit yield.

Levitt defined **elastic biological strain** as those changes in an organism's function that return to the optimal level when conditions are again optimum (that is, when biological stress has been removed). If the functions do not return to normal, the organism is said to exhibit **plastic biological strain**. The analogy with physical objects is clear: An elastic deformation strain in a steel bar, for example, disappears when the stress is removed; a plastic deformation does not (that is, the bar remains bent).

Plant physiologists have emphasized such plastic strains as those caused by the stresses of frost, high temperature, limited water, or high salt concentrations. Elastic strains in plants, such as reduced photosynthesis in response to low light (it returns to normal with the return of high light levels), have been less studied by stress physiologists, although they must be extremely common and have been emphasized in studies of stress in animals.

Levitt (1972, 1980) distinguished between avoidance and tolerance (hardiness) to any given stress factor. In **avoidance**, the organism responds by somehow reducing the impact of the stress factor. For example, a plant in the desert might avoid the dry soil by extending its roots down to the water table. If the plant develops **tolerance**, on the other hand, it simply tolerates or en-

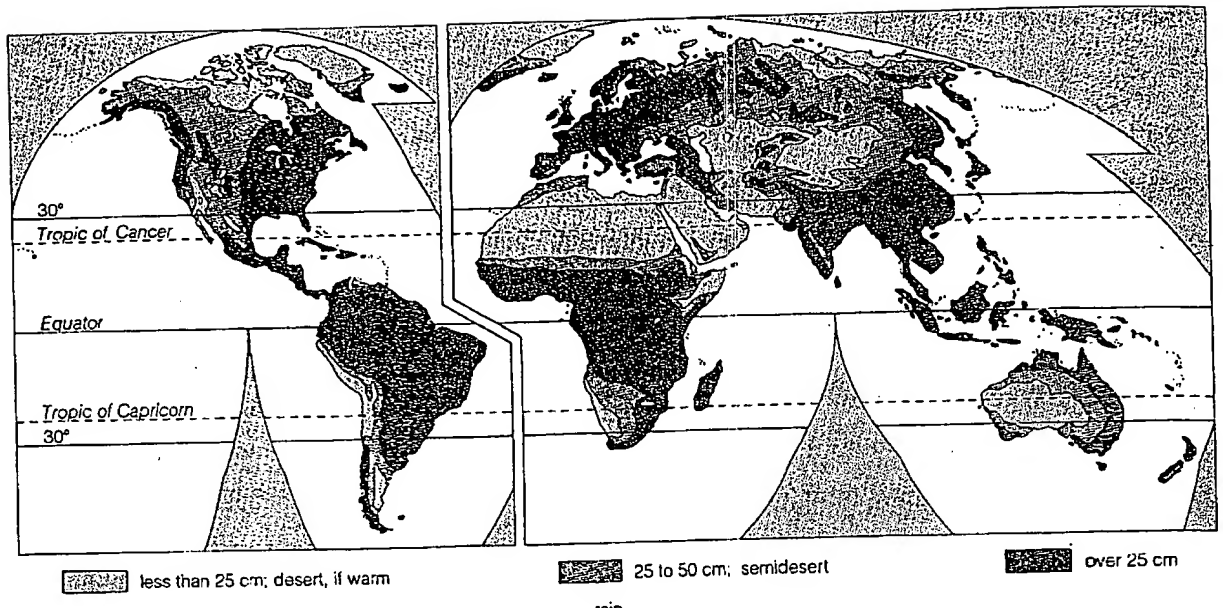


Figure 26-1 Map of the world showing areas of extremely low (less than 250 mm) and low (between 250 and 500 mm) precipitation (usually as rain). Horse-latitude deserts usually occur between north or south latitudes of about 20 to 30°. By far the most striking example is the Sahara Desert of North Africa, extending to the deserts of the Arabian peninsula and the Near East, but the horse-latitude deserts of Mexico, South America, South Africa, and especially Australia are also evident. Rain-shadow deserts are important in North America (the Great Basin northward through Canada to the Arctic), where they are caused by the Sierra Nevada, the Cascades, the Rocky Mountains, and other ranges. Rain-shadow deserts are also important in Central Asia (the Gobi Desert and others), where they are caused by the Himalayas and other mountain ranges. Note the low precipitation in the Arctic—in some areas as low as the Sahara and other extreme deserts. Because of the low temperatures in polar regions, evaporation is greatly reduced compared with warm areas such as the subtropics; hence more water is available for plant growth. Yet moisture as well as low temperatures can limit plant growth in the low-rainfall polar regions. (From Jensen and Salisbury, 1984.)

dures the adverse environment. Creosote bush is a good example of a desert plant that is drought-tolerant. It simply dries out but survives anyway; it tolerates or endures the dryness of its protoplasm.

For the most part, Levitt's definitions are based on concepts that have been developing for well over a century. The terms *avoidance* and *tolerance* were often used early in the 20th century (for example, by Shantz, 1927), but the derivation of the concepts of *stress* and *strain* from their physical counterparts has not been widely accepted by stress physiologists, and the terms have sometimes been criticized (for example, by Kramer, 1980). The problem is that the term *stress* has often been used in the sense of biological *strain* as defined by Levitt. We introduce the terms because they emphasize the difference between the cause (the stress) and the effect (the strain).

Walter Larcher (1987) at the University of Innsbruck in Austria noted that we can keep this distinction clearly in mind if we use certain modifiers for the term *stress*: **stress factor** = Levitt's stress and **stress response** = biological strain. Larcher pointed out that Levitt's concept works best when we are dealing with individual stress factors, although stress responses are typically caused by more than one stress factor (Larcher et al., 1990). Hot summer weather, for example, may produce stress factors of high light levels (photodestruction of chlorophyll), low humidity, dry soil, and high temperatures. Furthermore, stress responses are typically complex, are exhibited by various parts of the plant, and may involve such *stress hormones* as abscisic acid (ABA) and ethylene, which are distributed throughout the plant.

In the 1930s, Hans Selye (see Selye, 1936, 1950) developed a concept of stress in human medicine. In his lexicon, *stress* was a syndrome of reactions within an organism in response to one or more stress factors—and any environmental factor could act as an agent of stress when it deviated from its optimum level for the organism, as we have noted already. Larcher (1987) sug-

gested that we should speak of a state of stress when we use the term in Selye's sense. Selye's work emphasized the dynamic nature of stress responses in animals, and in many cases the stages of development of a state of stress can be applied to plants as well. When the stress factor is first experienced, there is the **alarm reaction**, in which the function of interest deviates markedly from the norm. Then comes the **resistance stage** (or **restitution phase**), in which the organism adapts to the stress factor and the function often returns toward its normal state (but may not completely achieve it). Finally, if the stress factor increases or continues for a long time, the **stage of exhaustion** may be reached, in which the function may again strongly deviate from the norm; this can eventually lead to death.

One more difficulty with the stress concept needs to be mentioned. Many plants found in what seem to be the most stressful conditions on earth—hot deserts, salty soils, or high mountaintops—often appear healthy and to be species not found anywhere else. If they are flourishing and apparently can't survive under other "less stressful" conditions, is it valid to think of them as being stressed? Actually, many such plants grow better under less stressful conditions if they have the chance. It has been suggested that they normally do not occur in more moderate situations because they cannot compete with the plants already growing there (Barbour et al., 1987). They are stressed in their native habitats in the sense that energy must be expended to overcome the harmful effects of the stress factors (Larcher, 1987)—for example, to pump salts into the vacuole from the cytoplasm, where they could denature enzymes. For that matter, as we saw in the essay on page 560, studies with canopies of wheat in controlled environments suggest that crops even in the most productive fields are stressed; it is possible to create environments in which they will yield more (but not *much* more on an individual-plant basis).

26.2 Stressful Environments

Recalling our discussion of the operational environment (Section 25.2; Spomer, 1973), we realize that an environmental stress means that some potential in the environment differs from the potential within the organism in such a way that there is a driving force for transfer of energy or matter into or out of the organism that could lead to a stress response. Low outside water potentials, for example, provide a driving force for loss of water; low temperatures can lead to loss of heat. But what are the environmental limits for the existence of life on our planet? We might look for answers by examining those environments in which productivities are lowest and thus the stresses might be the greatest.

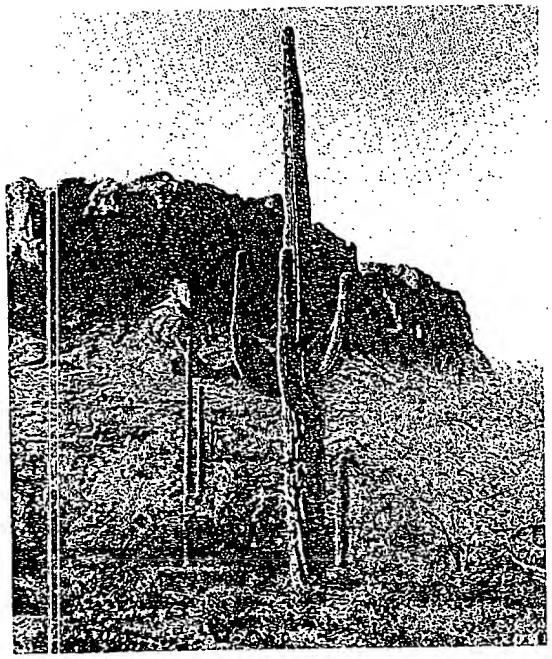


Figure 26-2 The desert east of Phoenix, Arizona, on the northern edge of the horse latitudes. A giant saguaro (pronounced sah-WAH-ro) cactus dominates the picture, with a much smaller cholla (CHAW-yuh) at its base and to the left. Numerous desert shrubs dominate the vegetation; the Superstition Mountains are in the background. The photograph was taken in mid-July. (Saguaro is *Cereus giganteus*; cholla is an *Opuntia* species, a common desert genus with many species. Photograph by F. B. Salisbury.)

Deserts and Other Dry Areas

A desert (Fig. 26-1) is an area of low rainfall—an area of **drought**—with less than about 200 to 400 mm of precipitation per year, depending upon temperatures, potential for evaporation, season of precipitation, and other factors. Deserts often have sparse but fascinating vegetation (Fig. 26-2). The most extensive deserts occur in the so-called **horse latitudes**, which range from approximately 20 to 30° north and south of the equator (30 to 35° over oceans). In these regions, air that has ascended in other latitudes descends and is thereby compressed and warmed, forming a zone of high pressure. Warm air holds more moisture than cold air, so precipitation does not occur, and the descending air does not produce many surface winds.[1]

[1]Reportedly, the horse latitudes received their name because horses died in the becalmed ships and had to be thrown overboard.

North of the northern horse latitudes (and on the southern tip of South America, south of the southern horse latitudes) deserts also occur. Global air movements in the northern and southern temperate zones are predominately from west to east (the **westerlies**). Storm systems moving this way in the northern hemisphere rotate in a counterclockwise direction (clockwise in the southern hemisphere), so a storm center is preceded by south winds and followed by north or west winds. As storms approach a mountain range, the rising air expands and cools and can then hold less moisture, resulting in precipitation on the western slopes, which are typically covered with lush forests. On the eastern slopes, the air descends, compresses, warms, and can hold more moisture. Areas east of the mountains have low precipitation and are called **rain-shadow deserts** because they occur in the "rain shadows" of mountains. The descending warm and dry winds on the eastern slopes of the Rockies are called **chinooks** or snow eaters. North and east of the Alps, such winds are called the **Föhn**. The deserts of the Great Basin occur in the rain shadow east of the Sierra, but the plains east of the Rocky Mountains are not rain-shadow deserts because they receive moisture moving north from the Gulf of Mexico.

Deserts in the horse latitudes are typically hot and dry all year, whereas most rain-shadow deserts are cold during winter. Because air above deserts is usually dry, it absorbs relatively little incoming sunlight or outgoing long-wave thermal radiation. Thus deserts are hot during the daytime and relatively cold at night. Air warmed in desert valleys rises, whereas air that cools at higher elevations flows down canyons and gullies, especially during the night. Hence, wind is common and can lead to dune formation, although the great sand dunes associated with deserts, as portrayed by moviemakers and others, are not as common as we are led to believe (except in the Sahara Desert). Perhaps so-called **desert pavement** is more common. This consists of a surface layer of small stones, finer material having been eroded away, mostly by wind.

Desert soils are often salty because the low rainfall does not leach away the salts as they form by weathering of soil particles and rock. The actual status of a desert soil depends considerably upon the time during the year when rain does fall. Mediterranean climate zones, for example, usually occur just to the north of the northern horse latitudes or to the south of the southern horse latitudes. The horse latitudes follow the sun and shift away from the equator during summer, so Mediterranean climates experience summer drought that can last from six to nine months. Yet they may have considerable rainfall during the winter months when the horse latitudes have shifted back toward the equator. Technically, their abundant winter moisture keeps these areas from qualifying as true deserts. Because of the high precipitation during part of the year, these soils are usually less salty than other desert soils—but at the same time, they have some of the characteristics of desert soils (for example, relatively high *p*H). Summer showers in the Arizona desert (moisture from the Gulf of Mexico, mostly) lead to a unique and relatively lush desert vegetation, but temperatures are high, as is evaporation, and total precipitation is low enough that these areas qualify as true deserts.

Actually, the uncertainties of world climatic patterns mean that some regions in the temperate zone that are normally blessed with ample rainfall for productive agriculture may experience droughts that extend for several weeks to months and/or reduced precipitation that may last for several years; examples are the dust bowls of the southern plains in the 1930s, the widespread 1988 summer drought in much of the United States, and the serious drought in Europe during the summer of 1983. Thus **water stress** (water potential negative enough to damage plants) occurs in many parts of the world besides deserts.

Tundras and Other Cold Areas

Tundras are areas on the earth's surface where temperatures are too low to permit the growth of trees (Fig. 26-3). Such areas occur on the tops of mountains (**alpine tundra**) and in the far north (**arctic tundra**). (Antarctic tundras are very limited in extent.) The polar tundras occur because the sun is relatively low in the sky even in summer (and actually below the horizon for days to months in winter poleward of the arctic and antarctic circles), so the slanting solar rays must follow a long pathway through the atmosphere and then strike horizontal surfaces at such acute angles that the energy of a given cross section of solar radiation is spread over a relatively large horizontal area. These combined effects (long atmospheric pathway and low solar angle) become increasingly important as one moves from the equator toward the poles and result in colder temperatures occurring at any given elevation. Stated another way, a given cold temperature occurs at lower elevations as the poles are approached. The **upper tree limit (tree line)**, for example, occurs at lower and lower elevations when one moves from the equator (where it is typically 3,500 to 4,500 m) until it reaches sea level some distance above the arctic circle. Alexander von Humboldt described this phenomenon in 1817; the principle is called **Humboldt's law**.

Why are temperatures low in alpine tundras? If a volume of gas expands or contracts without exchanging heat with its surroundings, the expansion or contraction is said to be **adiabatic**. As air rises, it expands because pressures are lower at higher elevations. If the expansion is adiabatic, the temperature of the air will decrease because there will be less heat per unit volume

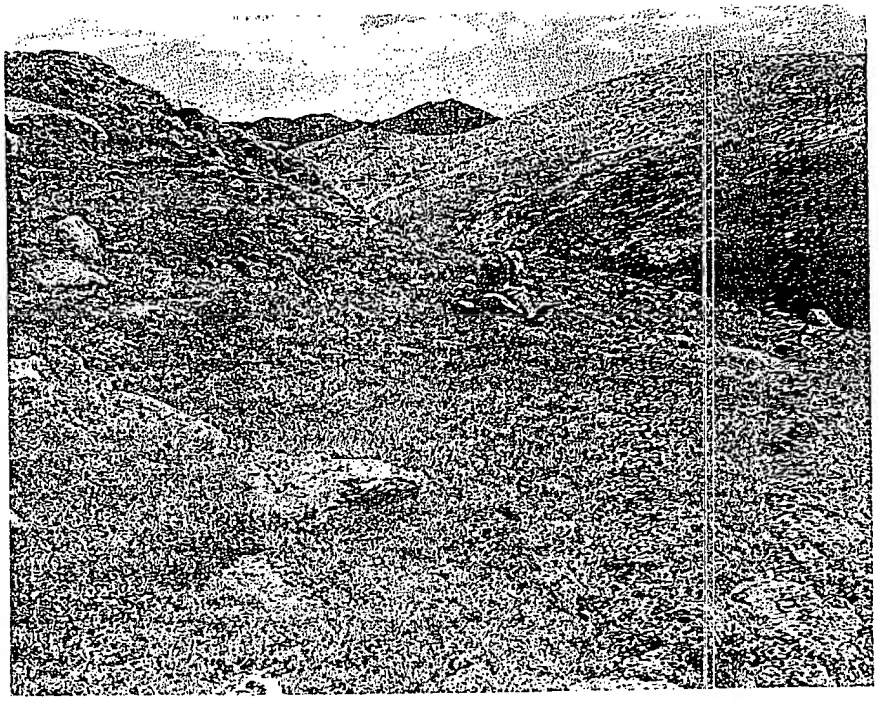


Figure 26-3 Alpine tundra high above tree line (note trees in distance at center right) on the northern border of Rocky Mountain National Park, Colorado, U.S.A., in the Mummy Range. The elevation is about 3,420 m (11,200 ft) above sea level, and the area was one of several sites for a physiological-ecology study of tundra plants. Because of different slopes, snow accumulation areas, substrates, and other features, several distinct vegetation types can be recognized in the tundra, but all are characterized by small plants, often with colorful flowers. (Photograph by F. B. Salisbury.)

of air. Dry air in the earth's atmosphere cools about 1°C for each 100 m of vertical rise. Thus, if dry air at 30°C on the valley floor is raised by global winds to a mountain top 1,500 m (4,921 ft) above, it will cool to about 15°C unless it is warmed or cooled by the mountainside or sunshine on the way up. This rate of cooling is an expression of the **adiabatic gradient**, or **adiabatic lapse rate**, for dry air in the earth's atmosphere (Fig. 26-4). Adiabatic cooling during expansion is the basic reason that higher air is nearly always cooler than lower air.

There is another important reason why alpine tundras are cool. At night any surface usually radiates more heat into the sky than radiates to it from the atmosphere above, so the surface cools. This is especially true for alpine surfaces because the atmosphere above is colder and thinner than it is above lower surfaces. So tundras cool faster at night than do lower areas. The cooled surfaces cool the adjacent air, which contracts, becomes more dense, and often flows down canyons or off slopes, replacing warmer air that is rising in the valleys.

During the day, high gusty winds are characteristic of alpine tundras, partly because of rapid temperature fluctuations but also because the mountain peaks deflect the global air movements that always occur high in the atmosphere. These winds increase evaporation from plants and soil, but humidity is usually high, which reduces evaporation. Virtually everything fluctuates rapidly in the alpine environment: wind velocity, temperature, light level (under partially cloudy skies, which are common), and humidity (see introduction to Chapter 21). The winter winds mean that much of the alpine tundra is blown free of snow, whereas deep snow drifts form in more protected areas. Some plants must thus endure the frigid temperatures of exposed areas while other plants avoid the winter extremes under a thick insulating blanket of snow.

High mountains near the equator have alpine tundras that do not experience winter (snow cover) but that can experience night temperatures as low as −6 to −11°C at any time during the year (Körner and Larcher, 1988; Sakai and Larcher, 1987). Actively growing plants living there must avoid or resist freezing the same as do plants in more temperate alpine tundras. These tropical tundras are characterized by a few succulent rosette plants, often with stems several meters tall (for example, species of *Dendrosenecio* and *Lobelia*).

Alpine and arctic tundras share similar average low temperatures but otherwise differ considerably in such factors as radiation flux, which is much lower in the arctic but is extended over longer days in summer. Light levels increase in alpine tundras because of a thinner layer of scattering atmosphere. Even when the sky is overcast, the diffused light measurable within clouds shrouding the tundra is much brighter than the light below clouds in the valleys. Highest irradiances are observed when the sky is partly cloudy, so that direct

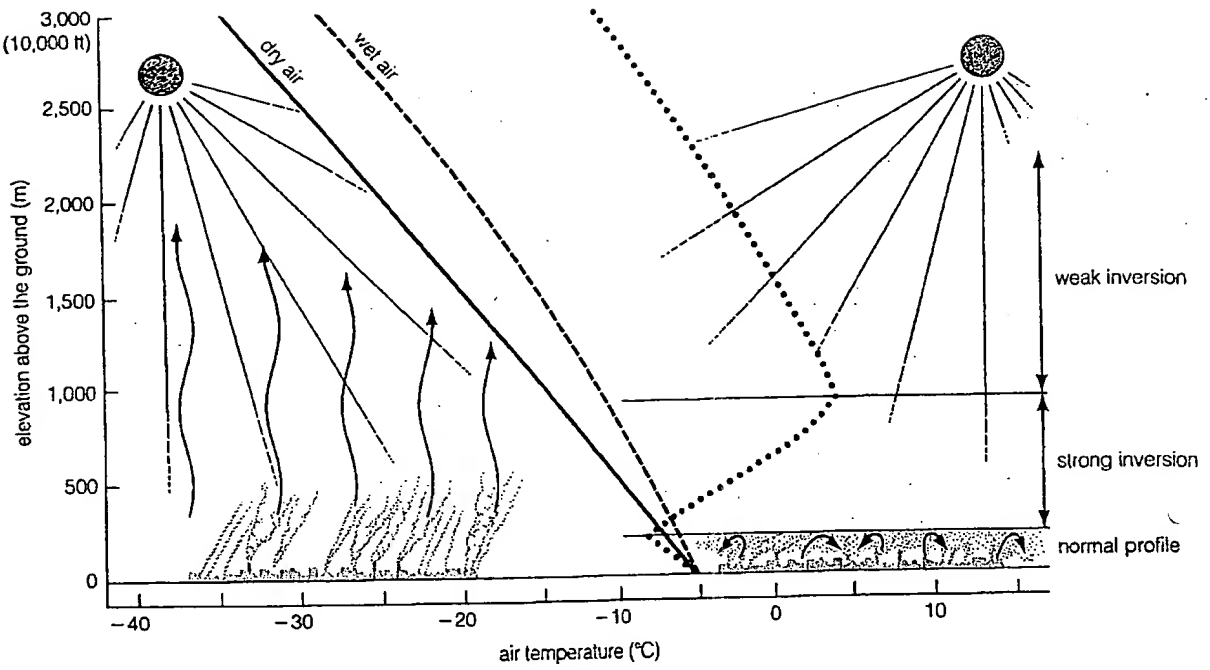


Figure 26-4 The adiabatic lapse rate and atmospheric inversion. The two dividing lines that angle to the left between the two parts of the figure show temperature profiles (temperature as a function of elevation) that are examples of the adiabatic lapse rates for dry and wet air. These show the temperature of a volume of air that is allowed to expand adiabatically (without exchanging heat with its surroundings) and thus to cool as it rises through the elevations shown. If the temperature gradient is steeper than the adiabatic lapse rate, as is usually the case, air warmed by contact with the warm ground rises by convection (left side of figure). If the gradient is less steep than the lapse rate, air does not rise by convection (right side). The dotted line shows an actual temperature profile measured with a sounding balloon on a cold December 31, 1963, above Salt Lake City, Utah. Below about 200 m, the temperature gradient was steeper than the lapse rate, and air rose—but only to the bottom of the layer of air in which the profile was actually reversed from its normal condition. The air was warmer instead of colder with increasing elevation up to about 900 m. This was an unusually strong inversion—and a collector of atmospheric pollutants. The inversion continued even above 900 m because the temperature gradient was still not as steep as the lapse rate, even for wet air. (From Jensen and Salisbury, 1984.)

rays of the sun and reflected rays from the clouds both fall on the same area. Irradiances may be as high as 1,500 W m^{-2} (solar constant = 1,350 W m^{-2}). Ultraviolet radiation can be high and perhaps important for organisms in alpine tundras, but it is very low in the arctic. The fact that tundra vegetation is so similar in alpine zones and in the arctic (often the same or closely related species but separate ecotypes; Section 25.4) testifies to the importance of low temperature.

What is special about the plants of the alpine or arctic tundras? Of course they are able to survive freezing, but why are they invariably small compared with their counterparts of warmer climates? Körner and Larcher (1988) noted that, contrary to widespread beliefs, the photosynthetic capacity of plants from cold regions is not essentially different from that of plants in temperate regions, when comparable life forms are considered. Prevailing leaf temperatures seldom limit seasonal photosynthetic carbon gain. Körner and Larcher suggested that cold affects tundra plants in two ways: indirectly, via the briefness of the growing season, and directly, via effects of reduced temperature on the developmental processes of growth, particularly cell division. Ecologists have largely overlooked the role of developmental processes in natural ecosystems, but tundra plants may well provide a logical example of the importance of such effects.

Having emphasized the low temperatures of tundras, we should note that plant leaves are not themselves always cold. Warmed by the sun, they may reach temperatures 20°C above air temperature (see, for example, Salisbury and Spomer, 1964; reviewed by Körner and Larcher, 1988). But temperatures are almost always low at night, and the growing season is very short. Daily

temperature fluctuations are often 25°C and may reach 50°C (Körner and Larcher, 1988).

We use the tundras as examples of regions on earth where low temperatures are especially important, but freezing temperatures are also important every year in temperate zones, and frost can be damaging to native and especially crop plants even in Mediterranean and subtropical climates. Even in the tropics, unusually low temperatures (although they may be as much as 10°C above freezing) can cause chilling injury to such sensitive plants as bananas.

Other Stressful Environments

There is a great variety of stressful environments on the earth's surface in addition to those we have described (Crawford, 1989). Some are of limited scale; others may be nearly worldwide in distribution. Flooding, for example, can produce a stressful condition quite opposite to the extremely negative water potentials of deserts, but damage results from the exclusion of oxygen rather than from the high water potentials. High temperatures, often close to or even above the boiling point, occur in hot springs, near volcanoes, in piles of decaying organic matter, and in extremely hot deserts, such as in Death Valley, California or Saudi Arabia. Living organisms often occur in these situations, as we shall see. We have already discussed (Chapters 12 and 25) stresses caused by low light levels in forests and at depths in bodies of water.

There are many spots on earth where soils are either highly acidic or highly alkaline, or where they are deficient in several nutrients (as are sand and some volcanic materials) or specific nutrients (especially nitrogen). The open oceans are deficient in many nutrients because these are carried to the ocean depths in the dead bodies of various organisms, especially **plankton** (single-celled plants and other organisms) as they settle to the bottom. Thus low nutrient concentrations (potentials) present special problems over the nearly two-thirds of the earth's surface covered with water; they are **nutrient deserts**.

The extreme pressures and total darkness that exist at great depths in the ocean, where organisms nonetheless live, surely seem stressful. Yet the organisms are alive and seem to be doing well (often existing on the "rain" of organic matter from above). The concept of stress that comes readily to our minds is a function of the environments to which we are ourselves adapted.

26.3 Water Stress: Drought, Cold, and Salt

Although there are many complications, a creosote bush growing in the desert, a white mangrove growing in a coastal forest with its roots in salt water, and a white spruce living in the north woods are all stressed, at least at certain times during the year, by a common factor: negative water potentials (water stress). We will begin our survey of this subject with the plants of the desert.

The Xerophytes

We can classify plants according to their response to available water: **Hydrophytes**[2] grow where water is always available, as in a pond or marsh; **mesophytes** grow where water availability is intermediate; and **xerophytes** grow where water is scarce most of the time. Solutes strongly influence water potential and can have specific toxicities, so ecologists further classify plants that are sensitive to relatively high salt concentrations as **glycophytes** and those that are able to grow in the presence of high salt concentrations as **halophytes**. (Such organisms that are not plants are called **halophiles**.)

All the plants of the deserts are called xerophytes, but different species survive the drought in various ways. Figure 26-5 expands the concepts of avoidance and tolerance introduced above. There are several forms of avoidance, but tolerance is always a matter of endurance. Homer LeRoy Shantz (1927) used four terms in classifying xerophytes. The terms are descriptive and are shown in Figure 26-5: *escape*, *resist*, *avoid*, and *endure*. Xerophytes in the desert are actually exposed to a wide range of water potentials. Plants such as the palms that grow at an oasis, where their roots reach the water table, or other plants such as mesquite (*Prosopis glandulosa*) and alfalfa (*Medicago sativa*) that have roots that extend as much as 7 to 10 m down to the water table, never experience extremely negative water potentials. They are *water spenders*. They certainly avoid the drought. Of course, such plants must be able to use the available soil water while they are extending their roots to the water table. (In the Judean Hills of Israel, certain trees have extended their roots down along tight fissures in solid limestone to depths of 30 m or more, dissolving the rock as they go.)

The so-called **desert ephemerals** are annual plants that *escape the drought* by existing only as dormant seeds during the dry season. When enough rain falls to wet the soil to a considerable depth, these seeds often germinate, perhaps in response to the leaching away of germination inhibitors (Chapter 22). Many of these plants grow to maturity and set at least one seed per plant before all soil moisture has been exhausted. They are eminently well suited to dry regions and thus are xerophytes in the true sense of the word, yet their active and metabolizing protoplasm is never exposed to extremely negative water potentials and is not drought-hardy. As with each group of xerophytes outlined here,

[2]The names are from Greek: *-phyte*, "plant"; *hydro*, "water"; *meso*, "middle"; *xero*, "dry"; *glyco*, "sweet"; and *halo*, "salt."

the ephemerals form a class that consists of many species, each with its own special characteristics and ways of responding to different amounts of water or nutrients (Ludwig et al., 1989).

Succulent species such as the cacti, century plant (*Agave americana*), and various other crassulacean-acid-metabolism (CAM) plants (Section 11.6) are *water collectors;* they resist the drought by storing water in their succulent tissues. Enough water is stored, and its rate of loss is so extremely low (because of an exceptionally thick cuticle and stomatal closure during the daytime), that they can exist for long periods without added moisture. MacDougal and Spaulding (1910) reported that a stem of *Ibervillea sonorae* (a desert succulent from Mexico in the cucumber family, the Cucurbitaceae) stored "dry" in a museum used stored water to form new growth every summer for eight consecutive summers, decreasing in weight only from 7.5 to 3.5 kg! Because their protoplasm is not subjected to extremely negative water potentials, succulents are drought avoiders and are not truly drought-tolerant. The water potential in their tissues is often about −1.0 MPa. Some of the succulents, especially the cacti, have extensive shallow root systems that absorb (collect) surface moisture after a storm, storing it in their succulent tissues.

Some species that are subjected to periodic drought can switch from CAM, which conserves water because stomates are closed during the day, to C-3 photosynthesis when water becomes available (see Section 11.6). *Clusia rosea* is a tree that begins as an *epiphyte* (seeds germinate on branches of other trees) in the rain forest. During the time between rain storms, the young plants may be seriously water-stressed because their roots are not in the soil. Schmitt et al. (1988) found that plants would switch from CAM to C-3–photosynthesis quickly in response to changing moisture and irradiance levels.

Many nonsucculent desert plants have other adaptations that reduce water loss; they are *water savers*. For example, it is common for desert shrubs and other plants to have small leaf blades. This condition increases heat transfer by convection, lowering leaf temperature and thus reducing transpiration (Sections 4.8 and 4.9). Such leaves will still be as warm as the air temperature, but air temperatures are seldom fatal. Other adaptations that reduce transpiration include sunken stomates, shedding of leaves during dry periods, and heavy pubescence on leaf surfaces (Ehleringer et al., 1976). It is also important that such plants increase root resistance to prevent water loss to dry soil (Schulte and Nobel, 1989). Although these modifications may indeed reduce the loss of water, they never completely prevent it and are by themselves insufficient protection against extreme drought.

As water evaporates from plants, salts in the protoplasm could reach levels that could damage crucial enzymes. An important adaptation found in many organisms subjected to water and other stresses is the accumulation of certain organic compounds such as sucrose, amino acids (especially proline), and several others that lower the osmotic potential and thus the water potential in cells without limiting enzyme function. As water stress increases, such compounds appear in the cells of many xerophytes (see Section 26.4); the resulting drop in osmotic potential is called **osmotic adjustment** or **osmoregulation** (Morgan, 1984).

Perhaps most impressive among the xerophytes are plants that simply endure the drought. They lose large quantities of water, so their protoplasm is subjected to extremely negative water potentials, yet they are not killed. Such **euxerophytes** (true xerophytes) exhibit *dehydration tolerance* or **hardiness** rather than mere avoidance. Plants that only avoid drought are of great interest to ecologists, but they do not challenge our physiological understanding to the degree that euxerophytes do. Incidentally, many characteristics of the drought avoiders, such as small leaves and sunken stomates, also occur in the drought endurers. Yet in the euxerophytes the ultimate weapon against drought is the ability to endure it—to be drought-tolerant.

The ability of some euxerophytes to endure drought is phenomenal. Water content of the creosote bush (*Larrea divaricata*), a desert shrub of both North and South America, drops to as little as 30 percent of the final fresh weight before the leaves die. With most plants, levels below 50 to 75 percent are lethal. Some of the most spectacular euxerophytes (called **poikilohydric** by some ecologists) are mosses and ferns—plants that we normally associate with wet environments. Their ability to dry out and then become metabolically active immediately upon rehydration apparently depends upon special features not common to other plants. Examples are *Selaginella lepidophylla* (the resurrection plant), certain grasses, and *Polypodium* (a fern).

Much work on desert xerophytes has been done in the Negev Desert of Israel. Researchers (Evenari et al., 1975) there have studied algae, lichens, and mosses that can tolerate extreme and prolonged desiccation, as well as extreme cold and heat when they are dry. They can take up water directly and instantaneously from dew, rain, or even a moist atmosphere (some when relative humidity is as low as 80 percent), and such water absorption leads to an instantaneous switching on of metabolic activity. (Air at 80 percent RH and 20°C has a water potential of −30 MPa.)

Israeli workers have identified among higher plants most of the features just discussed, plus a few others. An important adaptation, for example, is that of **heteroblasty**, the ability of a single plant to produce morphologically and physiologically different seeds. Such seeds have different germination requirements, so only a few seeds in a given crop germinate at any given

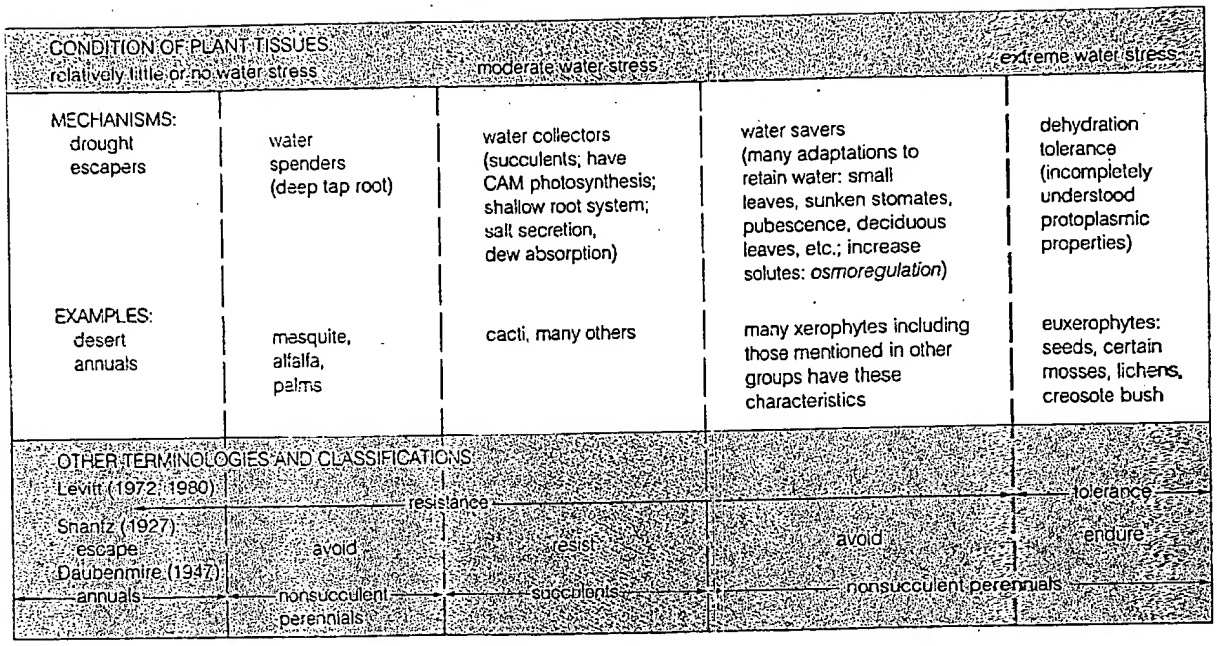


Figure 26-5 An approach to a classification of plant responses to water stress. Some other approaches are also shown.

time. The risk inherent in seedling survival is thus distributed over a variety of environmental conditions and sometimes over several years. These workers have also studied stomatal regulatory mechanisms in desert plants. In the most interesting examples, when water stress was low in the leaf, stomates opened when the temperature was raised; when water stress was higher and the temperature was again raised, stomates closed.

In the Negev Desert, plants often utilize dew. Other plants may also use dew, but the extent to which this contributes to plant growth remains somewhat controversial (Rundel, 1982). Later we'll consider an interesting example of salt secretion on leaves: The salt absorbs moisture from the air, and this water is then absorbed into the leaves.

Incidentally, insect physiologists (Edney, 1975) reported that certain insects absorb water from an atmosphere with a relative humidity as low as 50 percent—and thus a water potential of almost −100 MPa! Liquid within the insect's body might have a water potential of only −1.0 to −2.0 MPa and would be in equilibrium with an atmosphere of about 99 percent relative humidity. How absorption occurs remains a mystery.

There are numerous other adaptations of desert plants that cannot be discussed here for lack of space. For example, the **water-use efficiency** (the ratio of dry-matter production to water consumption) increases as soil-water availability decreases (Ehleringer and Cooper, 1988), and allelopathics (Section 15.3) are often produced by desert plants, restricting the germination or growth of competing plants, which reduces competition for water.

Water Stress in Mesophytes

Although desert xerophytes are studied by physiological ecologists, much work on water stress has been done by foresters and agriculturists (for example, Mussell and Staples, 1979). Water can limit crop growth and productivity virtually anywhere, either because of unexpected dry periods or normally low rainfall that makes regular irrigation necessary. When one must irrigate to obtain a crop, how much and how often should water be applied to the field? The answer, which could influence the expenditure of large sums of money, may be strongly influenced by research on stress physiology. Here we are seldom concerned with the severe stresses endured by desert plants; rather, we are interested in the extent to which withholding relatively small amounts of water might influence crop yield.

Extensive research has continued for well over a century. Thousands of papers on plant responses to drought have now been published. Many reviews and volumes that report on symposia have been published since 1980 (see, for example, Bewley and Krochko, 1982; Bradford and Hsiao, 1982; Greenway and Munns, 1980;

Table 26-1 Generalized Sensitivity to Water Stress of Plant Processes or Parameters.[a]

Process or Parameter Affected	Sensitivity to Stress: Very Sensitive → Relatively Insensitive; Tissue Ψ Required to Affect Process[b]: 0 MPa	−1.0 MPa	−2.0 MPa	Remarks
Cell growth	———	- - -		Fast-growing tissue
Wall synthesis	———			Fast-growing tissue
Protein synthesis	———			Etiolated leaves
Protochlorophyll formation	———			
Nitrate reductase level	———			
ABA accumulation	- - -	———		
Cytokinin level		———		
Stomatal opening	- - -	———	- - -	Depends on species
CO_2 assimilation	- - -	———	- - -	Depends on species
Respiration	- - -	———		
Proline accumulation		- - -	———	
Sugar accumulation			———	

[a]Length of the horizontal lines represents the range of stress levels within which a process first becomes affected. Dashed lines signify deductions based on more tenuous data.
[b]With Ψ of well-watered plants under mild evaporative demand as the reference point.

Source: From Hsiao, 1973.

Hanson and Hitz, 1982; Kramer, 1983; Levitt, 1980; Marchand, 1987; Morgan, 1984; Schulze, 1986; Staples and Toenniessen, 1984; Tranquillini, 1982; and Turner and Kramer, 1980).

Theodore Hsiao (1973; Bradford and Hsiao, 1982) has been especially active in this field. Table 26-1 (his 1973 summary table, which remains valid) outlines the sequence of events that occurs when water stress develops rather gradually as water is withheld from a plant growing in a substantial volume of soil. It is important to realize that the later events are almost undoubtedly indirect responses to one or more of the early events rather than to water stress itself.

Cellular growth appears to be the most sensitive response to water stress (Fig. 26-6). Decreasing the external water potential (Ψ) by only −0.1 MPa (sometimes less) results in a perceptible decrease in cellular growth (irreversible cell enlargement) and thus root and shoot growth (Neumann et al., 1988; Sakurai and Kuraishi, 1988). Hsiao suggested that this sensitivity is responsible for the common observation that many plants grow mainly at night when water stress is lowest. (But temperature, photoinhibition, and endogenous rhythms could also be involved.) The inhibition of cell expansion is usually followed closely by a reduction in cell-wall synthesis. Protein synthesis may be almost equally sensitive to water stress. These responses are observed only in tissues that are normally growing rapidly (synthesizing cell-wall polysaccharides and protein as well as expanding). It has long been observed that cell-wall synthesis depends upon cell growth (Section 16.2). The effects on protein synthesis are apparently controlled at the translational level, the level of ribosome activity.

At slightly more negative water potentials, protochlorophyll formation is inhibited, although this observation is based on only a few studies. Many studies indicate that activities of certain enzymes, especially nitrate reductase, phenylalanine ammonia lyase (PAL), and a few others, decrease quite sharply as water stress increases. A few enzymes, such as α-amylase and ribonuclease, show increased activities. It was thought that such hydrolytic enzymes might break down starches and other materials to make the osmotic potential more negative, thereby resisting the drought (osmotic adjustment), but careful studies don't always support this idea. Nitrogen fixation and reduction also decrease with water stress, a finding that is consistent

with the observed drop in nitrate-reductase activity. At levels of stress that cause observable changes in enzyme activities, cell division is also inhibited. And stomates begin to close, leading to a reduction in transpiration and photosynthesis.

There has been much controversy about whether the commonly observed drop in photosynthesis in response to only moderate stress is caused by stomatal closure or more directly by the water stress itself (see, for example, Kaiser, 1987). Calculations of the internal CO_2 concentration seemed to show that it remained high under moderate water stress, suggesting that photosynthesis itself was inhibited. But because the calculations were subject to several errors, this conclusion was questioned. Nevertheless, when the errors are taken into account, evidence remains suggesting that high transpiration rates can lead to decreased photosynthesis (Bunce, 1988). Measurements of responses of several photosynthetic enzymes to water stress also suggest a direct effect (Vu and Yelenosky, 1988). Final conclusions will depend on future research, and species differences will probably be important.

At about the level of stress that elicits effects on enzymes, abscisic acid (ABA) begins to increase markedly (at least 40-fold; see Sections 4.6 and 18.5) in leaf tissues and, to a lesser extent, in other tissues, including roots (reviewed by Bradford and Hsiao, 1982; Salisbury and Marinos, 1985; Walton, 1980). This leads to stomatal closure and reduced transpiration, as we discussed in Section 4.6. In addition, ABA inhibits shoot growth, further conserving water, and root growth appears (in some studies) to be promoted, which could increase the water supply. There is also evidence that suitably low concentrations of ABA increase the rate of water conductance through roots, which would reduce the water stress in the shoots. Most of these adaptations involving ABA are best observed in mesophytes; xerophytes often have other adaptations (Kriedemann and Loveys, 1974).

There is evidence that ABA normally plays a role in the resistance of mesophytes to water stress. Most studies have been done with drought-sensitive and drought-resistant cultivars of crop plants (see, for example, Quarrie, 1980). Often, resistant cultivars have higher levels of ABA when they are exposed to stress, and sensitive cultivars can be phenotypically converted to resistant types by application of ABA. But there always seem to be exceptions. A yellow lupine (*Lupinus luteus*), for example, is remarkably insensitive to applied ABA.

As a plant responds to water stress, what causes the increased ABA production in its tissues? Evidence suggests that lowered cell turgor is the trigger (reviewed by Bradford and Hsiao, 1982), but how turgor might control ABA synthesis remains unknown.

It is interesting that ABA increases in leaves in response to several kinds of stress, including nutrient deficiency or toxicity, salinity, chilling, and waterlogging. Reduced cell turgor and water potential are not involved in all of these. It may well be that ABA is a kind of universal stress hormone, its production controlled or triggered by several mechanisms. In all cases it seems to reduce growth and metabolism and thus conserve resources, which will then be available during recovery if and when the stress is removed.

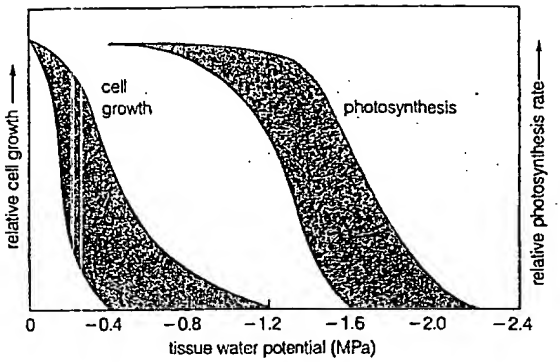


Figure 26-6 Cell growth and photosynthesis as a function of decreasing tissue water potentials. Shaded areas include ranges of response as observed with several species in different experiments. Cell growth (leaf enlargement, for example) is much more sensitive to decreasing water potential than is photosynthesis. (See Boyer, 1970; Acevedo et al., 1971.)

Ethylene also seems to play a role in stress reactions. It appears in response to various stress factors, including excess water (Jackson, 1985), plant pathogens, air pollution, root pruning, transplanting, handling (Section 19.2), and perhaps drought (Tietz and Tietz, 1982).

Although stresses are relatively mild at $\Psi = -0.3$ to -0.8 MPa, interactions and indirect responses begin to be the rule. Cytokinins decrease in leaves of some species at about these levels. At slightly more negative water potentials, the amino acid proline begins to increase sharply, sometimes building up to levels of 1 percent of tissue dry weight. Increases of 10- to 100-fold are common. Depending on species, other amino acids and amides, especially betaine, also accumulate when the stress is prolonged. Proline arises *de novo* from glutamic acid and ultimately probably from carbohydrates. These compounds contribute to osmotic adjustment (discussed below).

At higher levels of stress ($\Psi = -1.0$ to -2.0 MPa), respiration, translocation of assimilates, and CO_2 assimilation drop to levels near zero. Hydrolytic-enzyme activity increases considerably, and ion transport can be slowed. Actually, in many species respiration often increases, not really dropping off until water stresses of -5.0 MPa are reached.

Plants usually recover if watered when stresses

are −1.0 to −2.0 MPa, meaning that, in spite of the severity of the water stress, the stress response was elastic—or at least somewhat elastic, because growth and photosynthesis in young leaves frequently do not reach the original rates for several days, and old leaves are often shed. Clearly, because growth is especially sensitive to water stress, yields can be noticeably decreased with even moderate drought. Cells are smaller and leaves develop less during water stress, resulting in reduced area for photosynthesis. Furthermore, plants may be especially sensitive even to moderate drought during certain stages, such as tassel formation in maize. Ultimately then, in the sense of final yield, stress responses are really plastic, even with moderate water stress.

Salt Stress

A common and important stress factor in deserts is the presence of high salt concentrations in the soil (reviewed by Flowers et al., 1977). Soil salinity also restricts growth in many temperate regions besides deserts (Greenway and Munns, 1980). Millions of acres have gone out of production as salt from irrigation water accumulates in the soil. A plant faces two problems in such areas, one of obtaining water from a soil of negative osmotic potential and another of dealing with the high concentrations of potentially toxic sodium, carbonate, and chloride ions. Some crop plants (for example, beets, tomatoes, rye) are much more salt-tolerant than others (for example, onions, peas), and many crops have cultivars that are relatively salt-tolerant.

In the study of salt tolerance, the **euhalophytes** (*true* halophytes that tolerate or endure high levels of salt) are particularly interesting. Several such species grow best where salt levels in the soil are high, as in deserts or in soils saturated with brackish waters on the sea coasts or close to the shores of extremely salty waters such as the Great Salt Lake, where the salt content may be saturated at levels as high as 26 percent by weight.[3] They also grow in nonsalty soils. *Allenrolfea* (iodinebush), *Salicornia* (pickleweed or samphire), and *Limonium* (sea lavender, marsh rosemary) are representative genera. Species of *Atriplex* (shadscale) and *Sarcobatus* (black greasewood) grow in somewhat less salty soils, and certain bacteria (archaebacteria?) and blue-green algae (cyanobacteria) live in the waters of the Great Salt Lake. In general, prokaryotes and archaebacteria are more resistant to environmental stresses than are eukaryotes.

Barbour (1970; see also Barbour et al., 1987) reviewed the literature that suggests that *no* angiosperms

[3]During the early 1980s, several wet years caused the volume of the Great Salt Lake to quadruple; its salt content dropped to about 7 percent.

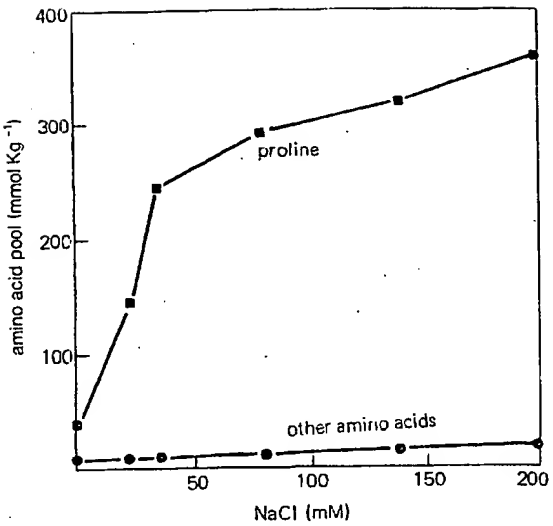


Figure 26-7 Amino acid and proline accumulation in *Triglochin maritima* grown at different salinities. Cuttings of *T. maritima* were grown in a nonsaline medium for two weeks before being transferred to saline media. Shoot tissues were harvested for analysis after 10 days of saline treatment. The extent of proline accumulation in this specialized halophyte is extreme compared with most plants, but accumulation of proline or other compatible solutes as drought or salt stress increases is common. Values in stressed mesophyte leaf tissues seldom exceed 200 mmol kg^{-1} of dry mass. (Note that values in this figure are for fresh mass; from Stewart and Lee, 1974.)

are **obligate halophytes**, plants that cannot grow unless the soil is salty. All halophytes studied so far have sometimes been found growing naturally in nonsalty soils and will grow well when planted in nonsalty soils. Normally, they are not abundant in nonsalty soils because they cannot compete with the glycophytes that normally grow there. As we discuss below, however, members of the genus *Halobacterium* (prokaryotes, again) accumulate large amounts of salt into their cells and cannot survive except in salty environments.

In terrestrial halophytes, the osmotic potential of leaf cell sap is invariably highly negative. Sap from tissues of actively growing *Atriplex* species, having no special cold hardiness, for example, freeze only when temperatures drop below −14°C, implying that their osmotic potentials are as low as about −17.0 MPa. This contrasts with a normal −1.0 to −3.0 MPa in most plants. In some cases, the xylem sap does not have a highly negative osmotic potential but may be almost pure water. To obtain water from the surrounding soil, the water potential within the xylem sap must then be greatly lowered by tension. This was demonstrated by Scholander and his coworkers for mangrove trees (see Fig. 5-17).

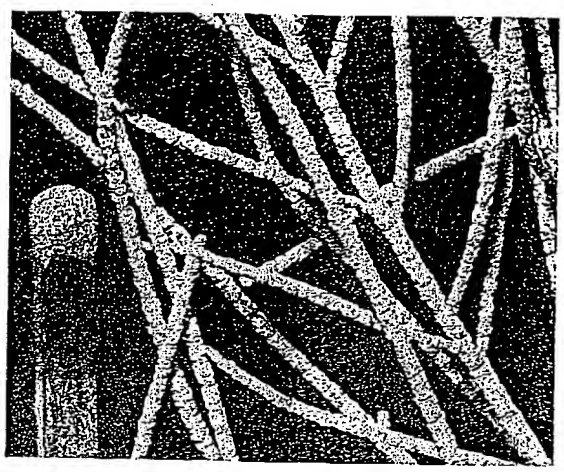


Figure 26-8 Tamarisk (*Tamarix pentandra*) leaves collected at Barstow in the Mojave Desert, California, showing heavy incrustations of salt. The paper match indicates the scale. (Photograph by F. B. Salisbury.)

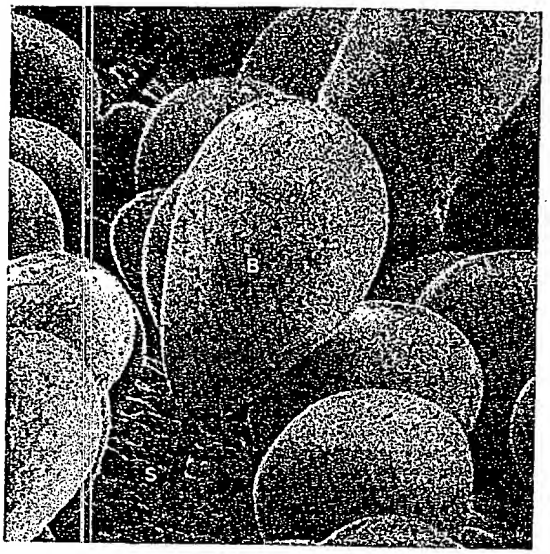


Figure 26-9 A scanning electron micrograph of salt bladders on the leaf of saltbush (*Atriplex spongiosa*). This species of saltbush is indigenous to Australia and is salt-tolerant because it has developed a mechanism to control the Na^+ and Cl^- concentrations of its tissues by accumulating salt in epidermal bladders on the surface of the aerial parts of the plant. Salt from the leaf tissues is transferred through the small stalk cell (S) and into the balloonlike bladder cell (B). As the leaf ages, the salt concentration in the cell increases, and eventually the cell bursts or falls off the leaf, releasing the salt outside the leaf. 450 ×. (From Troughton and Donaldson, 1972.)

Some halophytes are referred to as **salt accumulators**. In these species (for example, *Atriplex triangularis*; Ungar, 1977) the osmotic potential continues to become more negative throughout the growing season as salt is absorbed. Even in these plants, however, the soil solution is not taken directly into the plant. Based upon quantities of water transpired by the plant, it is easy to calculate that if the complete soil solution were absorbed, the plant would contain 10 to 100 times as much salt as is actually observed. Instead, water moves into the plant osmotically and not simply in bulk flow. The endodermal layer in the roots probably constitutes the osmotic barrier.

Halophytes in which the salt concentration within the plant does not increase during the growing season are known as **salt regulators**. Salt-tolerant derivatives of wheat, for example, limit the accumulation of Na^+ and Cl^- ions under salt stress compared with sensitive cultivars (Schachtman et al., 1989). Mangroves provide another spectacular example, excluding nearly 100 percent of the salt (Ball, 1988).

Often salt does enter the plant, but because the leaves swell by absorbing water, concentrations do not increase very much, if at all. This leads to the development of **succulence** (a high volume/surface ratio), a common morphological feature of halophytes. Ice plant (*Mesembryanthemum crystallinum*) is a good example (see Flowers et al., 1977). Rapid growth is another mechanism that dilutes the salt. In these cases, and when salt is excluded by the roots as in mangroves, organic compounds without the toxic effects of salt build up in the tissues, maintaining osmotic balance with the soil solution. Proline is a common example (Fig. 26-7), but other amino acids and such other compounds as galactosyl glycerol and organic acids also occur (Hellebust, 1976). As we shall see in a subsequent section, these compounds function in osmotic adjustment.

Sometimes excess salt is exuded on the surface of the leaves, helping to maintain a constant salt concentration within the tissue (Fig. 26-8). In certain halophytes there are readily observable salt glands on the leaves, sometimes consisting of only two cells (Fig. 26-9). Although Na^+ ions are essential for some salt-tolerant species, it is probable that sodium is transported out of the cytosol by counter transport with incoming H^+ (see Section 7.10). This moves much of the ion out of the cytoplasm of both root and leaf cells, inwardly to the central vacuoles and outwardly to the extracellular spaces.

Nolana mollis, a dominant, succulent shrub of the Atacamba Desert of northern Chile, grows where rainfall is less than 25 mm y^{-1}, although high fog and a relative humidity around 80 percent are common. The plant is almost always wet to the touch. Mooney et al. (1980) found that salt glands on the leaves secrete salt (mostly NaCl) that absorbs water hygroscopically from the atmosphere. If the leaves are washed with distilled

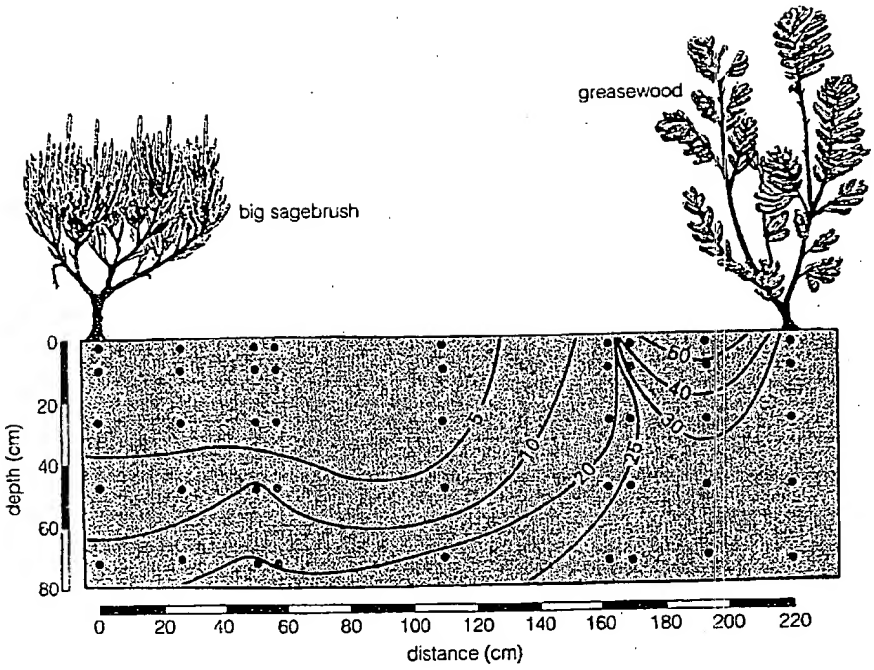


Figure 26-10 Salt concentrations in the soil (numbers in lines represent percent exchangeable sodium) as a function of position under a sagebrush plant (*Artemisia tridentata*) and under a greasewood plant (*Sarcobatus vermiculatus*) in the Escalante Desert of southern Utah. Note high sodium under greasewood. Dots are sampling points. (Data from Fireman and Hayward, 1952.)

water and blotted dry, they remain dry until they have had a chance to secrete more salt. If filter paper is soaked in solution collected from the leaves (or in concentrated NaCl solution) and then dried in an oven, it also absorbs water from the moist atmosphere and becomes wet to the touch. Can the plant absorb the water on its leaves? Mooney and his coworkers suggest two pathways of absorption: directly into the leaves or through the roots after the salty solution has dripped onto the ground. Either pathway would require the expenditure of metabolic energy by mechanisms that are not known to exist in plants, although they do exist (but are not understood) in insects and arachnids. The researchers calculate that ample respiratory energy is available, if a mechanism exists for its use.

Actually, large quantities of both organic and inorganic materials are leached from the leaves of many plants, both halophytes and glycophytes. Some of the leaching brought about by washing the leaves is caused by removal of materials within the tissues as well as washing off materials that have been exuded at the surface. In any case, materials that are washed from the leaves to the soil, or that fall with the leaves, are recycled back to the plant and to other plants. Species that absorb large amounts of salt and then lose it may considerably increase the salinity of the surface soil. Thus, as in other environments, desert plants sometimes profoundly influence the soil upon which they grow. Fireman and Hayward (1952) found, for example, that greasewood (*Sarcobatus vermiculatus*) in the Escalante Desert of Utah brought salts from depths, depositing them on the surface (Fig. 26-10), probably as leaves fell and decayed. The result was high salt concentrations beneath the greasewood plants, especially when compared with soil beneath big sagebrush (*Artemesia tridentata*) plants, which did not redistribute salt in this manner. Clearly, physiological differences between these two species are of considerable ecological significance.

Another potential problem for plants growing on saline soils is obtaining enough potassium. This problem exists because sodium ions compete with the uptake of K^+ by a low-affinity mechanism (Chapter 7), and K^+ is commonly present in such soils in much lower concentrations than is Na^+. The presence of Ca^{2+} appears to be crucial. If sufficient calcium is present, a high-affinity uptake system having preference for transport of K^+ can operate well, and the plants can then obtain sufficient potassium and restrict sodium (LaHaye and Epstein, 1969). It is possible that calcium fertilization of some saline soils low in Ca^{2+} might in-

crease their agricultural productivity. A favorable effect of Ca^{2+} on soil structure could also be important. Gypsum ($CaSO_4$) is sometimes used, providing both Ca^{2+} and some acidity, which helps in leaching out the Na^+. Elemental sulfur is also sometimes applied; it becomes oxidized to produce sulfuric acid, which aids in Na^+ leaching. Sulfuric acid itself has been applied with some success.

There has been considerable interest during recent years in the mechanisms by which Ca^{2+} overcomes the harmful effects of Na^+ (Cramer et al., 1985, 1986, 1988). For example, growth of maize roots was highly sensitive to NaCl (75 mM) in the nutrient solution, but this could be completely overcome by addition of Ca^{2+} (10 mM), *providing* that the Ca^{2+} was supplied *before* the sodium. This and other studies support the proposal that Ca^{2+} protects membranes from adverse effects of Na^+, thereby maintaining membrane integrity and minimizing leakage of cytosolic K^+.

A related line of research examines new polypeptides (proteins) that appear in response to salt stress (Hurkman et al., 1988; Ramagopal, 1987a, 1987b; Rouxel et al., 1989; see the discussion of heat-stress proteins in Section 26.6 below and the discussion of salt-stress proteins in Section 18.5). These proteins might be responsible for the membrane responses. A low-molecular-weight protein called *osmotin* seems to be especially important. Based on the observation that high Na^+ concentrations produced long and narrow cortical cells of cotton roots, Kurth et al. (1986) suggested that the Na^+/Ca^{2+} ratio might influence the deposition of microtubules in the cytoskeleton and thus the deposition of microfibrils in the wall. Iraki et al. (1989) studied the cell walls of isolated tobacco (*Nicotiana tabacum*) cells adapted to grow in strong salt solutions (for example, 0.428 M NaCl). In such solutions, cells were only one-fifth to one-eighth the volume of unadapted cells. The walls of the adapted cells were much weaker than normal walls because carbon was diverted from wall synthesis to formation of molecules important in osmotic adjustment (see Section 26.4 below).

In the context of studies on the role of membranes in plant stress responses, we note that many workers are currently attempting to understand the mechanisms of ion transport across membranes and that these mechanisms typically involve proton pumps (H^+-ATPases) that establish H^+ gradients across the membrane, which gradients can be used to drive solute uptake into plant cells through various secondary transport mechanisms. Calcium often plays a crucial role in the models that are developed (see Chapter 7 and such references as Butcher and Evans, 1987 and Giannini and Briskin, 1989). Blumwald and Poole (1987) showed that increased Na^+ in the growth medium of salt-tolerant cells from sugar beet induced a doubling in activity of Na^+/H^+ antiport activity at the tonoplast.

The Lower Temperature Limits for Survival and Growth

There is apparently no lower temperature limit for survival of spores, seeds, and even lichens and certain mosses in the dry condition. Such test objects have been held within a fraction of a degree of absolute zero for several hours with no apparent damage. Even active tissue may survive these low temperatures if it is experimentally cooled so rapidly that intracellular water freezes into extremely small crystals that don't damage the cytoplasm. But the lower temperature limits for survival under more normal circumstances depend strongly on the species and the extent to which the tissues have been hardened against frost—as we shall see in Section 26.4. Actively growing plants often can survive only a few degrees below 0°C, whereas many hardened plants can survive to about −40°C, and a few acclimated plants (willows and conifers, for examples) seem to have no low-temperature limits for survival (Sakai and Larcher, 1987).

What are the low-temperature limits for active growth as compared with survival? Several higher plants are able to grow and even flower under the snow, where the temperature is close to 0°C or sometimes below (Richardson and Salisbury, 1977). Such plants include native species such as the snow buttercup (*Ranunculus adoneus*), which forms flowers under snowbanks in the high mountains, as well as winter cereals (for example, winter wheat and winter rye) and ornamentals (crocuses, snowdrops, tulips, hyacinths, and daffodils—the last three not necessarily actually growing under snow). These are called **geophytes** or **spring ephemerals**. They grow slowly during the winter and thus often have a significant head start when the snow melts. The native species, especially, then grow rapidly and flower before later species overtop them or before trees above them leaf out.

It has been reported that lichens can photosynthesize at −20 to −40°C. Kappen (1989) reviewed these reports and questioned the data because the lichens might have been warmed to above the nearby air temperatures by radiation and may not have been totally frozen. Kappen's own measurements, carried out in Wilkes Land, Antarctica, detected vigorous photosynthesis at −10°C when the thalli had been sprayed with water before freezing. Frozen lichens covered with snow also exhibited active photosynthesis. Certain bacteria can grow at temperatures on the order of −22°C. Snow algae grow at the freezing point of pure water and below (Aragno, 1981). Actively growing giant rosette plants (*Dendrosenecio* sp. and *Lobelia telekii*) above tree line on equatorial mountains can experience night temperatures as low as −13°C at any time. Bodner and Beck (1987) found that such plants could photosynthesize when their cell water was supercooled to −8°C. When cells were dehydrated by freezing, photosynthesis

stopped, but it resumed immediately after thawing, a feature not seen in most flowering plants. Similar results were found with *Rhododendron ferrugineum* in the Austrian Alps in autumn (Larcher and Nagele, 1985). The lower limits for active growth of organisms have not been determined.[4]

Frost and Freezing Injury

Although productivity of world ecosystems is probably limited more by water than by any other environmental factor, low temperature is perhaps most limiting to plant distribution (Parker, 1963). To grow even in subtropical regions subject occasionally to freezing or even to near-freezing temperatures, plants must be capable of some acclimation to low temperatures. Plants that grow in polar regions must tolerate extremely low temperatures; only a few species can achieve this. Frost and chilling damage in crop plants is an important hazard nearly everywhere.

We might expect that death of a plant results from water expansion upon freezing and subsequent disruption of cell walls and other anatomical features. Careful examination during the early decades of the 19th century showed, however, that plants actually contract rather than expand upon freezing. This is because ice crystals grow into the extracellular air spaces. Furthermore, although it must occur, ice is almost never observed within the living cells of tissues that have frozen naturally. (Ice is, however, observed in the dead xylem cells of trees in winter; see Section 5.5.) Nor is damage to cells other than collapse observed. There is no rupture of cell walls or even of cell membranes, although there is ample evidence that membranes are damaged during thawing (Steponkus, 1984; for a recent scanning-electron-microscope study, see Pearce, 1988). With rapid tissue cooling in the laboratory (for example, 0.3 to 5°C min^{-1}), however, ice forms within the cells, and cellular components are damaged.

Such rapid cooling does occur in nature. Acclimated American arborvitae (*Thuja occidentalis*) tissues were capable of withstanding temperatures to −85°C when cooled slowly, but southwest-facing foliage was injured when the temperature dropped 10°C per minute from 2 to −8°C at sunset. Such changes duplicated in the laboratory also injured plants. Injury symptoms could not be duplicated by any form of desiccation, and it was concluded that the winter burn was caused by the rapid temperature drop (White and Weiser, 1964). Nevertheless, Sakai and Larcher (1987) questioned the conclusion that intracellular freezing is the cause of sunscald on south- and southwest-facing tree trunks. They suggested that the damage typically occurs in early or late winter before hardiness has had a chance to develop or after hardiness has begun to disappear.

Typically, ice crystals begin to form in the extracellular spaces, and water from within the cells diffuses out and condenses on the growing ice masses, which may become several thousand times as large as an individual cell. The cell acts as an osmotic system, with the osmotic concentration inside increasing as water diffuses out through the plasmalemma, dehydrating the cell. When these ice crystals melt in frost-hardy plants, the water goes back into the cells and they resume their metabolism. In nonacclimated plants, damage to membranes and other cellular components may have occurred, so metabolism cannot be resumed and the water does not reenter the cells completely. In the next section, we summarize what is known about the nature of frost damage and how plants can become resistant to it.

Hardening (Acclimation)

We have noted that plants can become acclimated to various stress factors by developing tolerance (becoming hardy) against the stress factor that induced the change and often against other stress factors as well. For example, plants exposed to low water potentials, high light levels, and such other factors as high-phosphorus and low-nitrogen fertilization become drought-tolerant (hardy) compared with plants of the same species not treated this way. Such acclimation to drought is of considerable importance to agriculture. It is a good example of a conditioning effect.

Actively growing plants, especially herbaceous species, are damaged or killed by temperatures of only −1 to −5°C, but many of these plants can be acclimated to survive winter temperatures of −25°C or lower. In regions where air temperatures drop below this, many plants have underground meristems protected from extreme air temperatures by soil or snow (Fig. 26-11). They avoid or escape cold rather than being extremely cold-hardy. Most species that survive freezing temperatures tolerate some ice formation in their tissues. Generally, hardier plants survive with more of their water frozen than do less hardy plants. But there are apparently several mechanisms of hardiness (see excellent discussions in Burke et al., 1976; Levitt, 1980; Sakai & Larcher, 1987; and Steponkus, 1981, 1984).

In practical terms, minor increases in hardiness could have a major impact on world food production. Winter wheats and winter rye yield 25 to 40 percent

[4]One of us (Salisbury) attended a NASA-supported Conference on Environmental Extremes held in San Diego, California, on February 10–11, 1966. There it was stated by experts in the field that certain bacteria grow at −22°C (as we have noted), that molds have exhibited active growth in cold-storage lockers at −38°C, and that spores were formed by these organisms at −47°C! We are currently unable to document these claims, and they should be viewed with suspicion (but see Allen, 1965).

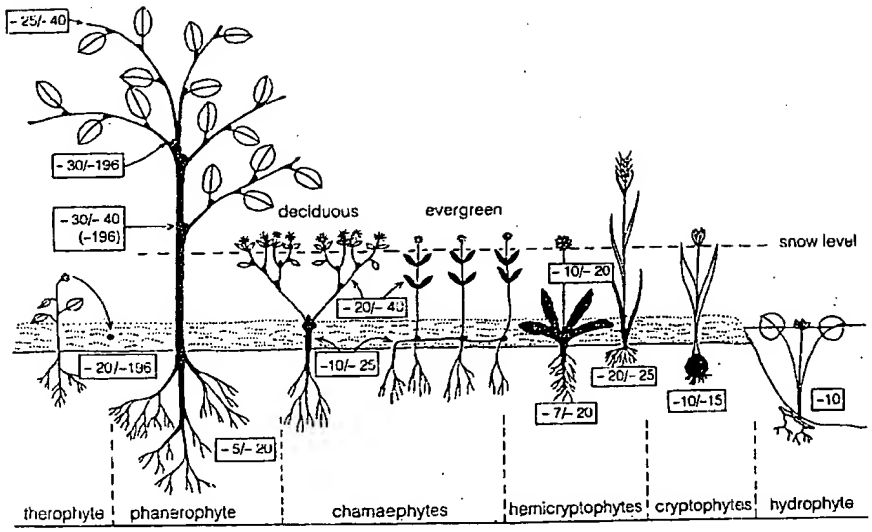


Figure 26-11 Typical temperature ranges of frost resistance for **life forms** according to Raunkiaer (1910). The overwintering parts are shown in black. (From Larcher, 1983; used by permission.)

more than comparable spring cultivars, for example, because they make better use of spring rains. If the winter wheats and rye could be made 2°C more cold-hardy, they could replace much of the large areas of spring wheats and rye in North America and the Soviet Union.

Frost hardiness typically develops during exposure to relatively low temperatures (for example, 5°C) for several days. Temperatures down to −3 to −10°C are sometimes required for maximum acclimation (Larcher and Bauer, 1981; Weiser, 1970). Short days also promote acclimation in several species, and there are indications that a stimulus may move from leaf tissue to the stems. The development of frost hardiness is a metabolic process requiring an energy source. Apparently this can be provided by light and photosynthesis. Factors that promote more rapid growth inhibit acclimation: high nitrogen in the soil, pruning, irrigation, and so on. In general, nongrowing or slowly growing plants are more resistant to several environmental extremes, including air pollution. Water-stressed plants are also more resistant to air pollution, partially because their stomates are closed.

Salt hardiness can be increased somewhat by exposure to saline conditions. Salt hardening is minimal, however, compared with drought or cold acclimation. Clearly, hardening against drought, freezing, and high salt concentrations is often a matter of hardening against water stress, but as we shall see in the next section, there are many complications.

26.4 Mechanisms of Plant Response to Water and Related Stresses

How does a euxerophyte differ from an ordinary mesophyte? Or how does a halophyte differ from a glycophyte? What is special about plants that can survive extremely low temperatures? Many different proposals have been presented to account for tolerance and acclimation, and a few of these may be common to the three kinds of stress (all related to water stress) that we are discussing here. For example, protoplasmic viscosity usually increases with high water stress, often to the point at which the protoplasm becomes brittle. Euxerophytes maintain protoplasmic plasticity much better at a given water stress than do mesophytes, and the hydrolytic activity (breakdown of starch, protein, and so on) is also less noticeable in euxerophytes. To a certain extent, these features also appear in plants capable of surviving extremely low temperatures, but halophytes often approach the problem in somewhat different ways. Here are some possibilities.

Proposals About Responses of Mesophytes to Mild Water Stress

How are mesophytes damaged by mild water stress? At least five possibilities have been proposed. First, it is known that water activity (indicating its ability to enter

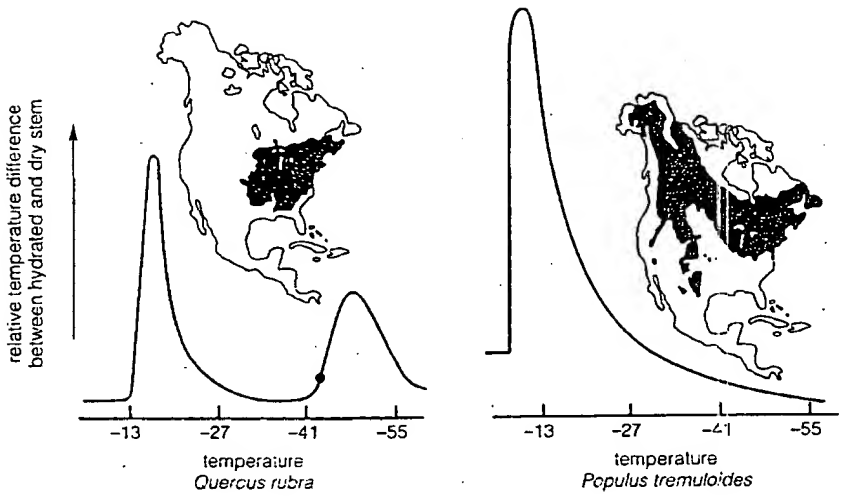


Figure 26-12 Differential thermal analysis (see also Fig. 26-13) of winter-hardy xylem and the natural ranges of northern red oak (*Quercus rubra*; deep supercools) and quaking aspen (*Populus tremuloides*; does not deep supercool). On the thermal-analysis curves (see Fig. 26-14 for explanation of such curves), the peaks to the left show the heat of fusion released when extracellular water freezes; the peak to the right in red oak shows the heat of fusion released when the supercooled sap inside of xylem parenchyma cells freezes. Aspen has no such peak and can withstand temperatures to −60°C. (From George and Burke, 1984; used by permission.)

into chemical reactions) is a function of water potential and is thus lowered by water stress. Nevertheless, water activity is closely related to water concentration, and as we have seen (Section 2.7), water concentration changes only slightly as water potential changes considerably. At the maximum stress levels of interest to agriculturists ($\Psi = -1.0$ to -2.0 MPa), water activity is lowered only slightly—probably not enough to be of any real consequence in chemical reactions. Second, solutes increase in concentration as water is lost. This could be important, but it is probably not very important under mild water stress simply because the concentration changes amount to only a few percent. Third, water stress might result in special changes in membranes. Such effects have indeed been demonstrated, but because comparable effects can be caused by other factors without noticeable plant response, it does not seem likely that this is an important aspect of plant response to water stress. Fourth, water stress might upset the hydration of macromolecules—the "ice" structure of the water molecules surrounding enzymes, nucleic acids, and so on. If this water of hydration is upset, function would also be influenced. Levitt (1962) has suggested that dehydration of key enzymes would cause disulfide bonds within proteins to break and reform, sometimes reforming between adjacent molecules, leading to enzyme denaturation when molecules are rehydrated. But again, it has been calculated that mild water stress would not have much influence on the structure of water of hydration, which involves only a small percentage of the water in a cell anyway. Amazingly enough, studies have shown that considerable water can be lost from a cell before enzyme function is noticeably influenced. But there could be exceptional enzymes that have not been studied.

Fifth, even the mildest water stress may profoundly change the turgor pressure within plant cells. Pressure changes of this magnitude ($P = 0.1$ to 1.0 MPa) probably have little effect upon most enzyme activities (judging by observed responses as well as thermodynamic principles), but such changes could be the stimulus to which some special response mechanism in the cell reacts in transducing water stress to the observed cellular responses. In the large-celled marine green alga *Valonia*, ion uptake decreases with slight increases in cellular turgor pressure. Such responses have not been observed in most higher-plant cells, although red-beet tissue responds this way to decreased turgor. The observation may serve as a model for what might be taking place (see discussion in Hellebust, 1976). We have already noted that ABA is apparently produced in response to decreasing leaf-cell turgor (Sections 4.6 and 26.3), and we have emphasized that cell expansion, meaning plant growth in general, is highly sensitive to water stress, probably via decreasing cell turgor (see Fig. 26-6).

Frost Damage and Frost Acclimation

Several good summaries review the status of our knowledge of frost effects (for example, Krause et al., 1988; Li, 1984; Marchand, 1987; Sakai and Larcher, 1987). Frost resistance is based either on tolerance to extracellular ice formation and thus severe cell dehydration (as in

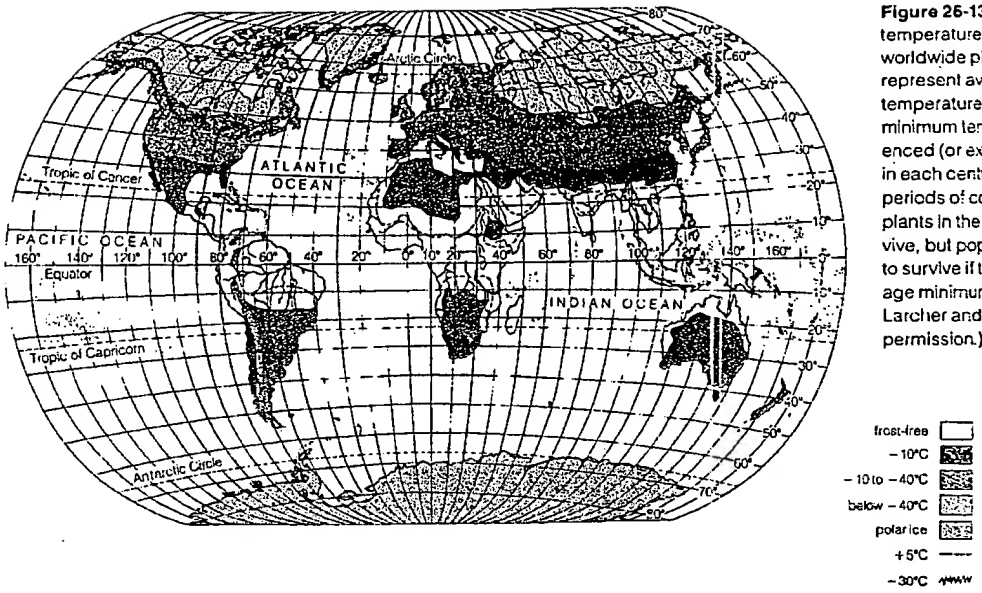


Figure 26-13 Tentative map of low-temperature thresholds limiting worldwide plant distribution. The lines represent average annual minimum temperatures rather than the lowest minimum temperatures ever experienced (or experienced once or twice in each century). When such unusual periods of cold occur, at least a few plants in the population usually survive, but populations cannot continue to survive if they cannot tolerate average minimum temperatures. (From Larcher and Bauer, 1981; used by permission.)

drought tolerance) or on frost avoidance, especially supercooling. We will discuss the dehydration effect first.

Although several significant differences in protoplasm have been observed between hardy and sensitive plants, most recent studies have emphasized either the compatible solutes (**cryoprotectants** in this context) that we discuss below or alterations in the various membrane systems in the cell, along with the patterns of proteins that are associated with these membranes (Gilmour et al., 1988; Guy and Haskell, 1987). The changed protein patterns also occur in response to other stresses, as we note below in the section called "Other Stress Proteins." There are also changes in growth regulators (especially ABA; Lalk and Dörffling, 1985), and Quader et al. (1989) observed reversible changes in elements of the cytoskeleton in response to cold stress.

There have been many suggestions about membrane changes in response to cold (Steponkus, 1984). For example, a low sterol/phospholipid ratio in the plasmalemma might stabilize the lamellar bilayer during freeze dehydration, but the evidence is contradictory (see Krause et al., 1988 for review). It is clear that in most species, freezing stress leads to an inhibition of photosynthesis after thawing and that the photosynthetic reactions of thylakoid membranes are temporarily impaired. Particularly, photosystem II is inhibited. Nevertheless, it appears that CO_2 assimilation is more sensitive to freezing stress than is activity of the thylakoids. Krause et al. (1988) reported that the light-regulated enzymes of the carbon reduction cycle appear to be the first to be affected. These enzymes reside in the chloroplast stroma.

Deep Supercooling and Ice Nucleation

Water in xylem tissues of most deciduous fruit trees and forest species, and in many dormant buds (see, for example, Ashworth, 1984), does not freeze until temperatures drop as low as about −40°C (lowest on record is −47°C). This phenomenon is called **deep supercooling** (Burke et al., 1976). Ice nucleation is relatively improbable in small water volumes about the size of a plant cell. Water can be supercooled to −38°C, and the solute-containing water in acclimated xylem cells apparently acts like water droplets in that the xylem cells are isolated from one another by air spaces, dry cell walls, and the plasmalemma. As in similarly acclimated herbaceous plants, ice forms in the bark and buds when temperatures are only a few degrees below the freezing point of pure water, but the crystals form in the spaces between cells, and tissues are not damaged by this. Xylem tissue in most hardwoods is too rigid and too impervious to water to permit the formation of such crystals, however. When freezing does occur, xylem ray parenchyma cells are killed, the wood becomes dark and discolored, and vessels are filled with gummy occlusions (Section 5.6). Wood-rotting organisms often invade such injured trees, which frequently die. Such species do not grow where winter temperatures drop below about −40°C (Figs. 26-12 and 26-13). This limit is set by the supercooling process; it proves to be the weak link in plants' survival.

Extremely hardy woody plants (for example, birches, alders, quaking aspen, willows) native to the boreal forests of the Northern Hemisphere do not undergo deep supercooling. Extracellular freezing oc-

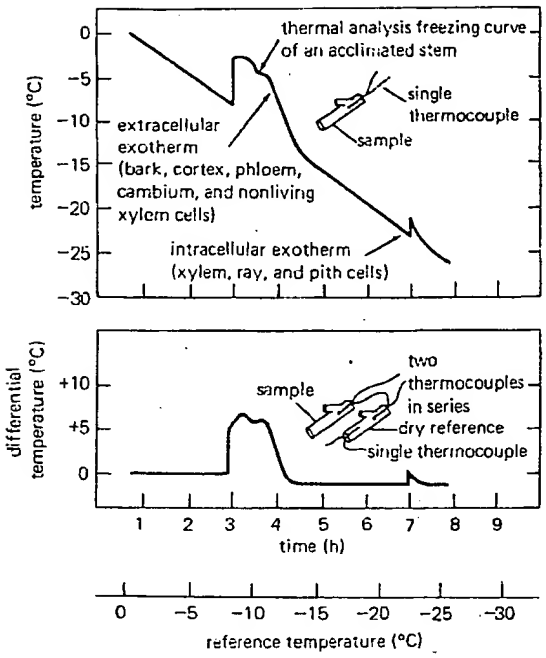


Figure 26-14 Thermal analysis and differential thermal analysis as methods to observe deep supercooling in stem samples. In both experiments the sample is cooled over a period of time, and the sample temperature (top) or the differential temperature between the sample and a dry reference (bottom; see also Fig. 26-12) is monitored. The peaks or *exotherms* show release of the heat of fusion and thus indicate freezing points. The first peak (left, warmest temperature) indicates freezing of extracellular water; the second peak (right, coldest temperature) presumably indicates freezing of water in cells. Cell freezing typically leads to the death of the cells. (From Burke et al., 1976.)

curs, and the cells are extremely tolerant of the dehydration of their protoplasm. As ice masses form, they draw water from the cells until all but the water of hydration (bound water) is removed. Such dormant, winter-hardy, woody plants (typically softwoods) can readily survive the −196°C of liquid nitrogen. These same plants, when actively growing, may be killed by −3°C! Thus cold acclimation is much more spectacular than drought or salt hardening.

Deep supercooling is observed by measuring stem temperatures with a small thermocouple device while temperature is lowered (Fig. 26-14). When the xylem ray cells finally freeze, the released heat of fusion causes a sharp temperature rise, as when osmotic potentials are determined cryoscopically (see Fig. 3-10).

Both tissue rigidity and submicroscopic anatomical features appear to be important in xylem supercooling (George and Burke, 1984). Membranes may play a role in the deep supercooling of living tissues by keeping ice nuclei from reaching the supercooled water. But water in wood with no living cells can also supercool, although not quite as much as in living wood. This suggests that the microstructure of cell walls can also separate supercooled water from ice crystals nearby. Apparently, as water leaves the cells to condense on the crystals, tension builds within the cells, lowering the water potential so that its vapor pressure remains in equilibrium with the ice. This model needs more testing, but it is supported by current evidence.

Plants with no appreciable level of hardiness may escape light frost damage by supercooling to −2 to −10°C. When they do freeze it is because of various things that provide nuclei for ice crystals. Sometimes these occur within the tissues, as has been shown for wood of *Prunus* spp. (Gross et al., 1988); other times they are on the surface of the leaves. Certain species of bacteria (for example, *Pseudomonas syringae* and *Erwinia herbicola*) have been found, for example, that initiate ice formation at relatively warm temperatures. Spraying frost-sensitive plants in the field with suspensions of these bacteria causes the plants to be killed by light frosts, whereas spraying with other bacterial species protected the plants by facilitating supercooling (Anderson et al., 1982; Lindow, 1983).

Compatible Solutes: Osmotic Adjustment (Osmoregulation)

We have noted in several places that certain substances such as proline, betaine, and various carbohydrates build up in cells that are subject to drought or high salt concentrations. This is called **osmotic adjustment** or **osmoregulation** (Flowers et al., 1977; Jefferies, 1981; Morgan, 1984; Turner and Jones, 1980).

Consider the situation with high salt concentrations. Because there are excessive dissolved salts in the soil solution (or sea water in the case of mangroves and other plants that grow in similar situations), the osmotic potential is negative enough to cause water to diffuse out of the tissues into the surrounding solutions—unless the water potential in the tissues is at least as negative. Actually, if the tissues are to absorb water and survive, their water potential must be more negative than that of the surrounding solution. One way to overcome this problem would be for the cells simply to accumulate salt to the same or higher concentrations as those outside the plant. Why doesn't this happen? (We noted that those plants that do accumulate salt usually dilute it by becoming succulent or accumulate the salt into their vacuoles, where it could still dehydrate the cytoplasm osmotically.)

In all eukaryotes so far studied, salts such as NaCl denature the enzymes and thus cannot be tolerated in the cytoplasm itself (although this effect may be somewhat less drastic than has been assumed; see Cheeseman, 1988). When they occur in halophyte cells, they

Table 26-2 Examples of Some Organisms and the Compatible Solutes That Increase in Their Cells During Osmotic Adjustment.

Organism	Compatible Solute
BACTERIA	
Various halophiles and nonhalophiles (e.g., *Klebsiella, Salmonella, Streptococcus*)	amino acids (glutamate, proline, etc.)
Halobacterium salinarium (halophile; an Archaebacterium; no osmotic adjustment)	NaCl
FUNGI	
Chaetomium globosum (a terrestrial form)	polyhydric alcohols (mannitol, arabitol, glycerol)
Saccharomyces rouxii (an osmophilic form)	arabitol
MICROALGAE	
Chlorella pyrenoidosa (freshwater)	sucrose
Dunaliella spp. (marine and halophilic)	amino acids, glycerol
Scenedesmus obliquus (freshwater)	carbohydrate (sucrose + raffinose, glucose, fructose)
ANGIOSPERMS	
glycophytes: *Chloris gayana, Hordeum vulgare* (barley)	betaine and proline
halophytes: *Aster tripolium, Mesembryanthemum nodiflorum, Salicornia fruticosa, Triglochin maritima*	proline
halophytes: *Atriplex spongiosa, Spartina townsendii, Suaeda monoica*	betaine

Source: Mostly from Flowers et al., 1977 and Yancey et al., 1982.

are on the order of 10 times more concentrated in the vacuole than in the cytosol. Apparently, many plants that tolerate the various kinds of water stress do so by synthesizing in their cytoplasm compounds that can exist at high concentrations without denaturing the enzymes essential for the metabolic processes of life. The organic compounds that can be tolerated have been referred to as **compatible solutes** (or **compatible osmotica**). Paul H. Yancey and his coworkers (1982) pointed out that the number of compatible solutes discovered among the five kingdoms of living organisms is relatively limited. Apparently only a few compounds can exist at relatively high concentrations in the cytoplasm without damaging the enzymes there. Table 26-2 lists a few species that exhibit different degrees of resistance to water stress (produced by drought, cold, or salt), along with the compatible solutes found in their cells. Figure 26-15 shows the chemical structures of a few of these compounds.[5]

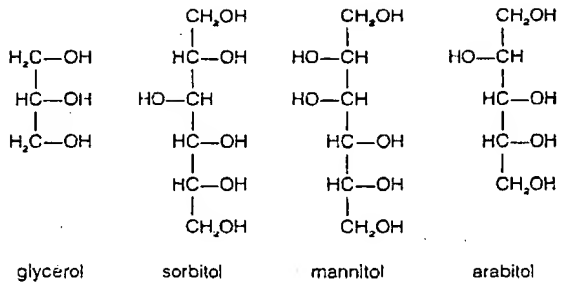
The degree of osmotic adjustment is a function of the degree of outside water stress caused by salt in the surrounding medium (see Fig. 26-10), by drying soil, and by frost hardening. The regulation or adjustment occurs in halophytes, xerophytes, and mesophytes.

[5]Roles in addition to that of compatible solute have been suggested for some of the compounds in Table 26-2. Proline, for example, might also serve in nitrogen storage and transport.

How does the regulation occur? Current research is concerned with this question, but complete answers remain to be determined (LeRudulier et al., 1984). We have already emphasized the role of cell turgor in synthesis of ABA and in controlling the rate of cell growth as it is influenced by water stress. Most workers in the field suspect that changes in cell turgor also activate and control the degree of compatible-solute synthesis. In the marine algae, for example, turgor pressure remains constant over a wide range of external salinities, indicating that it must be regulated and suggesting that cells are sensitive to changes in turgor. Perhaps the physical properties of the plasmalemma change as the force with which it is pressed against the wall changes. Felix D. Guerrero and John E. Mullet (1988) observed rapid changes in leaf translatable RNAs in response to a reduction in turgor of pea leaves. There is also the interesting observation that levels of putrescine, a polyamine, increased to over 60 times normal in oat-leaf cells within 6 h in response to osmotic stress (sorbitol and other osmotica dissolved in the media on which leaf segments were floated; Flores and Galston, 1982). Yet observed maximum concentrations of putrescine are still far too low for the compound to be acting as a compatible solute. Could the change be part of the transduction between cell turgor and the synthesis of such solutes? Future research is required to find out.

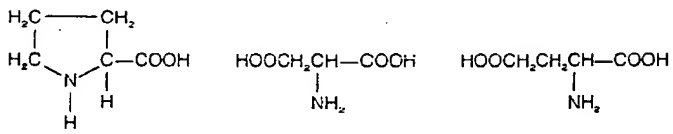
Membranes of organisms that produce compatible solutes must not only prevent the external salt from

polyols



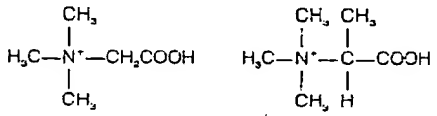
glycerol sorbitol mannitol arabitol

amino acids



proline aspartic acid glutamic acid

methylated quaternary ammonium compounds



betaine (glycinebetaine) alaninebetaine

Figure 26-15 Molecular structures of some compatible solutes found in stressed plants and other organisms. (See also Table 26-2.)

entering the cell but must also prevent the compatible osmoticum from leaking out. Gimmler et al. (1989) studied isolated plasma membranes of the extremely salt-tolerant unicellular green alga *Dunaliella parva*, which synthesizes large amounts of glycerol as a compatible solute. The membrane is not only highly impermeable to glycerol, but it has an effective ion-pumping capacity that actively exports the salt that leaks in. The plasma-membrane ATPases responsible for this pumping require unusually high concentrations of divalent cations (up to 100 mM Mg^{2+} or Ca^{2+}). Furthermore, fairly high concentrations (up to 800 mM) of NaCl or $NaNO_3$ (but not Na_2SO_4) were required for maximum activity of these enzymes, and the ATPases were extremely resistant to salt. About 2.5 M NaCl (about a 15 percent solution) was required for half-maximal inhibition of activity. Thus, the compatible solute (glycerol) in the cytosol of these cells allows the enzymes there to be more or less like their counterparts in glycophytes, but the enzymes in the plasma membrane, which are exposed to high salt concentrations, have the necessary adaptations.

There are a few organisms that seem to be the exception to the rule about compatible solutes; actually, they illustrate the importance of the rule. Certain species of *Halobacterium* (Table 26-2) accumulate large quantities of sodium chloride in their cells. These interesting organisms are considered by many bacteriologists to belong to a sixth kingdom, the Archaebacteria (or a third kingdom, if organisms are divided into Pro-

karyotes, Eukaryotes, and Archaebacteria). Some of these organisms are unable to grow and reproduce unless they are in highly concentrated salt solutions such as the Dead Sea or the Great Salt Lake. Whereas most enzymes of eukaryotic halophytes are identical (or nearly so) to their counterparts in glycophytic eukaryotes, the enzymes of halobacteria have been extensively modified. They maintain their metabolic activities only when they exist in strong salt solutions, being denatured by more dilute solutions. Thus, in an evolutionary sense it seems that the halobacteria have followed the extremely difficult pathway of modifying hundreds to thousands of enzymes so that they can tolerate high salt concentrations, whereas the other halophytes and salt-tolerant eukaryotes have taken the genetically much simpler approach of producing compatible solutes that do not harm cytoplasmic enzymes but provide a water-potential balance between the cytoplasm and the surrounding environment of high water stress (Yancey et al., 1982).

Although we have emphasized salt tolerance in our discussion of compatible solutes, it is important to note that these compounds also appear in many plants during frost acclimation, where they are called cryoprotectants. Indeed, increases in sugars and polyhydric alcohols during hardening were observed many years ago, although the correlation with hardiness was not always perfect (see the reviews in Krause et al., 1988; Sakai and Larcher, 1987). Free amino acids and such other substances as glycinebetaine and polyamines have also been observed. (Note that the term *osmoregulation* is usually not used with reference to cryoprotectants.)

26.5 Chilling Injury

Tropical or subtropical plants grown in southern regions of temperate North America are sometimes damaged by frost or even by temperatures slightly above freezing. Such crops include citrus, cotton, maize, rice, sorghum, soybean, sugarcane, and sweet potato. Certain tropical fruits such as bananas are damaged even by a few hours below 13°C. (Never put bananas in the refrigerator!) Timing is crucial: Rice plants exposed to temperatures below 16°C at the time of pollen-mother cell division will not produce a crop. It has been estimated that worldwide rice production would decrease 40 percent if worldwide mean temperature dropped only 0.5 to 1.0°C.

As in our discussion of water-stress effects, we are faced with the question of mild chilling effects (such as those that damage bananas), as contrasted to the death caused by severe freezing. Many mechanisms have been proposed to account for **chilling injury**. Because chilling disrupts all the metabolic and physiological processes in plants, it seemed almost futile to look for a single key reaction that might be responsible. Nevertheless, it is possible that just such a key response has been identified (Graham and Patterson, 1982; Lyons, 1973). As the temperature is lowered in chilling-sensitive plants, lipids in cellular membranes solidify (crystallize) at a critical temperature that is determined by the ratio of saturated to unsaturated fatty acids. This critical temperature for a phase transition from liquid to crystalline often proves to be equivalent to the temperature that causes chilling damage. Development of tolerance to chilling temperatures in chilling-sensitive plants apparently involves changes in this ratio. An increase in the proportion of unsaturated fatty acids or in the quantity of sterols causes the membrane to remain functional at lower temperatures.

The lipid model suggests that the membrane normally exists in a liquid-crystalline condition. In this state its enzymes have their optimal activity, and its permeability is thus under control. Below the critical temperature, the membrane exists in a solid-gel state. This change in state should bring about a contraction resulting in cracks or channels that lead to increased permeability (Yoshida et al., 1979). This would lead to the observed upset of solute balances (leakage from chill-damaged cells or organelles of ions and other solutes; also upset of proton transport; see DuPont and Mudd, 1985). Enzyme activities would also be upset, leading to imbalances with nonmembrane-bound enzyme systems. Thus, metabolites such as those produced in glycolysis would be expected to accumulate because they could not be acted upon by the mitochondrial enzyme systems. Such accumulation of glycolysis intermediates has indeed been observed.

It has been suggested that little ATP would be formed because of the importance of membranes in its formation and because of these imbalances between the mitochondria and the glycolytic systems. Similar events would probably take place between the chloroplast and the cytoplasm around it. Kasamo (1988) studied ATPases in the plasmalemma and tonoplast of chilling-sensitive and chilling-insensitive cultured cells of rice (*Oryza sativa*). Clearly, the activities of the ATPases were dependent upon the status of the membranes as influenced by temperature. Neuner and Larcher (1990) used *in vivo* chlorophyll fluorescence techniques to detect differences in chilling susceptibility of two soybean cultivars; again, photosynthetic membranes were involved (see also Larcher and Neuner, 1989, and Larcher et al., 1990).

If the temperature is raised soon enough, the membranes return to the liquid-crystalline state (because this phase transition is completely reversible), and the cell recovers. If metabolite buildup and solute leakage are allowed to occur to any great extent, however, cells are injured or killed.

Some cultivars are more sensitive to chilling than are others, although their fatty-acid ratios appear to be the same. These differences could be caused by different sensitivities to the accumulated metabolites rather than to the initial effects on the membranes. Chilling effects can often be avoided if tissues are exposed to high temperatures for brief intervals between chilling periods, provided that initial chilling was not too prolonged. In terms of the hypothesis given, this would allow the metabolism of accumulating metabolites, and so toxic levels are not allowed to develop.

In summary, there is much support for the lipid-membrane (with associated proteins) hypothesis for chilling injury, and the basic idea might even be extended to gain at least a partial understanding of frost hardening (Harvey et al., 1982; Steponkus, 1984). But there are still problems to be solved, mostly lack of correlation between lipid compositions and chilling sensitivity in some studies (summarized by Graham and Patterson, 1982).

26.6 High-Temperature Stress

Elevated temperatures typically accompany drought conditions and are an important environmental stress factor in themselves. This is especially true for euxerophytes that are hardly cooled by transpiration.

The Upper-Temperature Limits for Survival

Biologists have long been fascinated by growth of organisms at high temperatures (see, for example, Aragno, 1981; Brock, 1978; Kappen, 1981; and Steponkus, 1981). Plants typically die when exposed to temperatures of 44 to 50°C, but some can tolerate higher temperatures. Stem tissues near the soil line of plants in the desert, for example, may reach levels considerably above this, and *Tidestromia oblongifolia* photosynthesizes optimally in Death Valley, California, at these temperatures (see Fig. 12-4). *Stipa* spp., *Carex humulis*, and *Bothriochloa ischaemum* also can survive temperatures of 65 to 70°C (Larcher et al., 1989).

A few bacteria (*Thermotoga* sp.) can grow in thermal springs, such as those in Yellowstone National Park, at temperatures up to 90°C (Pool, 1990). But the Archaebacteria live at the highest temperatures (Pool, 1990). Members of the genus *Pyrodictium* were found growing at temperatures up to 110°C in fields of hot, bubbling, sulfurous mud (**solffatara fields**) in a hydrothermal system off Isola Vulcano, Italy, and most recently, Archaebacteria of the genus *Methanopyrus* were found growing at that temperature in sediment samples taken by the research submersible *Alvin* at the Guaymas Basin hot vents in the Gulf of California, at depths of 2,000 to 2,010 m (Huber et al., 1989). In the laboratory, the organisms grew best at 98°C but did not grow at 80°C or 115°C. They also grew best in 0.2 to 5 percent NaCl[6] (optimum, about 1.5 percent). Growth was strongly inhibited by traces of oxygen. At present, 110°C is the accepted upper temperature limit for growth of organisms.[7]

Such extreme conditions lead to denaturation of enzymes and unfolding of nucleic acids in most organisms. So how do the Archaebacteria manage? No one knows, but it has been suggested that special lipids occur in their membranes, that their proteins have some special structure (although their amino acids are the same as in other organisms), and that their DNA is protected by special histonelike proteins. When the proteins are added to DNA *in vitro*, it can withstand temperatures 30°C higher than usual (Pool, 1990).

Eukaryotic organisms have not been found growing at temperatures above 56 to 60°C (temperatures of hot springs that support green algae), and the upper limit for animals seems to be about 45 to 51°C. Photosynthesis apparently does not occur even in blue-green algae (prokaryotes) at temperatures above 70 to 72°C. Several dry spores and seeds of higher plants will survive temperatures well above 100°C, but these do not actively grow at such temperatures. In general, dry and dormant structures withstand various stresses well.

The deleterious effects of high temperatures on higher plants occur primarily in photosynthetic functions, and the thylakoid membranes, particularly the photosystem II complexes located on these membranes, are apparently the most heat-sensitive part of the photosynthetic mechanism (Santarius and Weis, 1988; Weis and Berry, 1988). Long-term acclimations can be superimposed on a more rapid adaptive adjustment to high temperatures that occurs within a few hours. In addition to the heat effects on primary photochemical reactions, there is evidence that rubisco and other enzymes of carbon metabolism are adversely affected. Energy dissipated by photorespiration can exceed that consumed by CO_2 assimilation. Light and other factors can

[6]Some Archaebacteria are known to live in saturated salt solutions (about 36 percent), in highly alkaline (*p*H 11.5) or acidic (*p*H of 1 or less) waters, and at the extreme pressures of ocean depths (1,300 to 1,400 atm; Pool, 1990).

[7]As we noted in the third edition of this text, J. A. Baross and J. W. Deming (1983) found Archaebacteria in hot water rising from sulphide-encrusted vents located along tectonic rifts and ridges of the deep ocean floor. Some communities occurred at temperatures exceeding 360°C! They claimed that they grew these bacteria at 250°C in a titanium syringe pressurized to 265 atmospheres (26.85 MPa) and containing enriched sea water. Unfortunately, the work was not duplicated by other researchers, and Baross and Deming have lost their cultures and are now unable to extend the work. They do not retract their claim, however.

cause an increase in tolerance to heat, but plants' acclimation to high temperatures is minimal (a few degrees) compared with acclimation to drought or to freezing temperatures. For example, soybean seedlings exposed for 2 h to 40°C are subsequently able to survive an otherwise lethal 2-h exposure to 45°C. This small acclimation could nevertheless be significant because high-temperature extremes may exceed normal high temperatures by only a few degrees. Many of the changes that appear during acclimation to heat stress are reversible, but if the stress is too great, irreversible changes can occur, and these can lead to death.

Plants that are hardy to high temperatures exhibit high levels of water of hydration and high protoplasmic viscosity, characteristics that are also exhibited by euxerophytes. High-temperature-adapted plants also are able to synthesize at high rates when temperatures become elevated, allowing synthetic rates to equal breakdown rates and thereby avoiding ammonia poisoning. These features were observed long ago, and some of them may be functions of the heat-shock proteins discussed in the next paragraphs.

Heat-Shock Proteins

During the past two decades there has been considerable interest in the so-called heat-shock response (see, for example, Key et al., 1985; Kimpel and Key, 1985; Lindquist and Craig, 1988; Ougham and Howarth, 1988; Sachs and Ho, 1986; and Section 24.5). Organisms ranging from bacteria to humans respond to high temperatures by synthesizing a new set of proteins, the **heat-shock proteins (HSPs or hsps)**; typically, synthesis of most of the normal proteins is repressed. The HSPs are studied by electrophoresis on SDS gels and by other methods. Some of these HSPs have relatively high molecular weights (for example, a 70-kilodalton HSP is common in many organisms, including plants), but a group of 20 to 30 low-molecular-weight HSPs (15 to 27 kDa) may be unique to plants. The HSPs appear rapidly, often becoming a substantial portion of the total proteins within 30 min after an abrupt shift from, say, 28°C to 41°C. Their synthesis continues during the next 3 to 4 h, but after 8 h the pattern of synthesis is essentially the same as it was at the initial low temperature. The HSPs also appear when the increase in temperature is more gradual, as might occur under natural conditions. Three or four hours after return to the normal temperature, HSPs are no longer produced, but many of the HSPs are still present, indicating that they are quite stable. Heat-shock mRNAs have also been studied. The kinetics of their appearance and disappearance matches that of the HSPs in the expected way.

It is becoming apparent that the HSPs play a role in heat tolerance, perhaps by protecting essential enzymes and nucleic acids from heat denaturation. Without such protection, nucleic acids might be cleaved by specific metal ions that leak into the cytoplasm from outside (or from the vacuole) as membranes become more permeable at the higher temperatures (Burke and Orzech, 1988).

Other Stress Proteins

Special proteins appear in response to stresses other than high temperatures (Sachs and Ho, 1986). Soybean seedlings treated with 50 to 75 μM arsenite for a few hours developed tolerance to a subsequent heat treatment and produced a pattern of proteins very similar to the HSPs of this species (Key et al., 1985). Water stress induces changes in protein and mRNA patterns, but these do not necessarily match the patterns of HSPs (Bray, 1988; Bensen et al., 1988; Ramagopal, 1987a, 1987b; and Ranieri et al., 1989). Bhagwat and Apte (1989) studied proteins induced in a nitrogen-fixing cyanobacterium (*Anabaena* sp.) by heat shock, salinity, and osmotic stress. They found 15 new polypeptides, four of which were unique to heat shock. Four others were induced by all three stresses. Michalowski et al. (1989) studied the time course for induction of mRNA in response to salt stress in the ice plant as the plant shifted to CAM metabolism. Special proteins formed in response to heavy metals and to ultraviolet light have also been noted (Sachs and Ho, 1986).

There have been several reports of **cold-acclimation proteins (CAPs)**; for example, Gilmour et al. (1988) found a pattern of new polypeptides (four 47-kDa, a 160-kDa, and others) that appeared in cold-hardened plants of *Arabidopsis thaliana*. The significance of the CAPs remains to be discovered, but Hincha et al. (1989) showed, in accordance with earlier work, that on a molecular basis these proteins are several orders of magnitude more efficient than sucrose in protecting thylakoid membranes from freezing damage.

26.7 Acidic Soils

Plants are found growing on soils in a *p*H range of at least 3 to 9, and the extremes provide another stress to which some species are adapted. Cranberries, for example, grow on acid bogs, whereas certain desert species normally grow only on soils with high *p*H. In general, we know far too little about why some plants are native to low-*p*H soils and others are native to soils with higher *p*H values. Certainly one of the reasons is competition. If we use hydroponic techniques to study the growth of various species that apparently prefer different *p*H levels, we usually find that they do reasonably well over a wide *p*H range. But in nature, even a slight advantage of one species over another can eventually lead to elimination of the less well-adapted one.

Soil factors closely correlated with *p*H are probably more important than the concentration of H^+ ions *per se*. For example, high rainfall leads to leaching of calcium and formation of acidic soils, so calcium is usually low in acidic soils and abundant in soils of high *p*H (calcareous soils). Moderate concentrations of this element favor development of root nodules on many legumes (Chapter 14), so nitrogen-fixing legumes will grow better on soils rich in calcium than on most acidic soils. The less-abundant calcium in acidic soils may also limit plant growth simply because H^+ is much more toxic to roots in the absence of calcium. One of the beneficial effects of liming acid soils no doubt derives from this fact. (Liming involves addition of calcium in various forms, often in mixtures: CaO, which is lime or burned lime; $Ca(OH)_2$, which is water-slaked or hydrated lime; or $CaCO_3$, which is limestone, dolomite, or air-slaked lime.)

The *p*H also strongly influences the solubility of certain elements in the soil and the rate at which they are absorbed by plants. Iron, zinc, copper, and manganese are less soluble in alkaline soils than in acidic soils because they precipitate as hydroxides at high *p*H. Iron-deficiency chlorosis is thus common on soils in the western United States, which are often alkaline. Phosphate, absorbed largely as the monovalent $H_2PO_4^-$ ion, is more readily absorbed from nutrient solutions having *p*H values of 5.5 to 6.5 than at lower or higher *p*H values. In soils of high *p*H, more of the phosphate is present as the less readily absorbed divalent HPO_4^{2-} ion. Furthermore, much of this is usually present as insoluble calcium phosphates. In soils of low *p*H, in which $H_2PO_4^-$ should predominate, the frequent high concentrations of aluminum ions cause its precipitation as aluminum phosphate.

The relatively high concentrations of available aluminum in many acidic soils (those below about *p*H 4.7) can inhibit growth of some species, not only because of detrimental effects on phosphate availability but apparently also by inhibiting absorption of iron and by direct toxic effects on plant metabolism. Some species (for example, azaleas) not only tolerate these high aluminum concentrations but thrive on such soils. Still other species tolerate amounts of various heavy metals that are toxic to most plants. An example is bentgrass (*Agrostis tenuis*) that is grown in Wales and Scotland on mine tailings having unusually large amounts of lead, zinc, copper, and nickel. This grass does not exclude such toxic metals but somehow accumulates them without being injured appreciably. We don't understand the tolerance mechanism, although it has been suggested that specific chelating agents (for example, in root cell walls) form strong complexes with the metal ions and prevent their reaction with sensitive protoplasmic constituents such as enzymes. Secretion of these metals into the vacuoles would also decrease their toxic effects. (See box essay in Chapter 6 on tolerance to heavy metals.)

26.8 Other Stresses

Although we have examined the most important environmental stress factors, there are still others that might be discussed. For example, certain species of plants not only survive but flourish on soils derived from serpentine rock (Kruckeberg, 1954; Whittaker, 1954) or highly acidic material derived from rock that has been strongly modified by percolation of hot water in ancient hot springs (Salisbury, 1964, 1985). The serpentine soils are very deficient in calcium and apparently have toxic amounts of other elements. Somehow serpentine species have adjusted to these stresses. Material derived from hydrothermally altered rock has most of its phosphate tied up in forms unavailable to most plants. There is little available nitrogen, whereas iron, aluminum, and calcium occur in superabundant amounts. Such conditions are not only stressful but fatal to many species; yet, again, a few species have adjusted to these stress factors. Often, plants from both serpentine and hydrothermally altered material can also grow well on more normal soils. Much needs to be learned about such situations.

If space permitted we could discuss at some length ultraviolet light as a stress factor. We have mentioned the topic in other chapters, including the final paragraphs of the previous chapter.

We are becoming acutely aware of the importance of atmospheric pollutants as stress factors. Because of the implications for agriculture, and for the health and productivity of the world's forests and other ecosystems as well, these stress factors have been the objects of much research in recent years. Often they form valid and important topics of plant physiology that we will arbitrarily not discuss, mostly because of space limitations.

In spite of the broad spectrum of environmental stress factors, it is proper to end this chapter and our textbook by returning to the importance of water in the life of most plants. Water stress is either of primary importance or is a contributing factor to reduced growth and yields of plants growing on much of the earth's surface.

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Arabidopsis thaliana AtHAL3: a flavoprotein related to salt and osmotic tolerance and plant growth

Ana Espinosa-Ruiz, Jose M. Bellés, Ramón Serrano and Francisco A. Culiáñez-Macià*
Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, 46022 Valencia, Spain

Summary

We have isolated two *Arabidopsis thaliana* genes, *AtHAL3a* and *AtHAL3b*, showing homology with HAL3, a yeast protein which regulates the cell cycle and tolerance to salt stress through inhibition of the PPZ1 type-1 protein phosphatase. Expression of *AtHAL3a* in yeast *hal3* mutants partially complements their LiCl sensitivity, suggesting possible conserved functions between both proteins. *AtHAL3a* and *AtHAL3b* are induced by salt stress and *AtHAL3a* is the most expressed in non-stressed plants, particularly in seeds. *In situ* hybridization demonstrates enrichment of *AtHAL3a* mRNA in seed embryos and in the vascular phloem of different plant tissues. AtHAL3 proteins show striking homology with a group of proteins found in fungi, plants and animals and some homology with a large family of prokaryotic flavoproteins. Recombinant AtHAL3a protein purified from *Escherichia coli* was yellow because it contained a non-covalently bound chromophore revealed as flavin mononucleotide. Transgenic *Arabidopsis* plants, with gain of *AtHAL3a* function, show altered growth rates and improved tolerance to salt and osmotic stress.

Introduction

Abiotic stresses, particularly drought and salinity, account for major losses in the yield of crop plants (Boyer, 1982). Improving plant traits that allow better adaptation to adverse environmental conditions is a challenge for modern plant biotechnology. Plant molecular biologists and breeders should co-operate in working towards preventing desert land expansion and world starvation.

One approach to drought and salt stress is to determine pathways, genes and metabolites involved in the complex plant response to these stresses (Bray, 1997; Ingram and Bartels, 1996). Another strategy is the isolation of plant genes homologous to those that play a fundamental role in improving drought and salt tolerance in lower organisms (Serrano, 1994; Holmberg and Bülow, 1998).

We were interested in the isolation and characterization of an *Arabidopsis thaliana* gene homologous to the yeast *Saccharomyces cerevisiae SIS2/HAL3* gene (referred to as *HAL3*) (Di Como *et al.*, 1995; Ferrando *et al.*, 1995). Overexpression of *HAL3* improves growth of wild-type cells exposed to toxic concentrations of sodium and lithium. Although the sequence of HAL3 gives little clue about its function, alterations in intracellular cation concentrations associated with changes in *HAL3* expression indicate that HAL3 activity directly increases cytoplasmic K^+ concentration and decreases Na^+ and Li^+ concentrations (Ferrando *et al.*, 1995). In addition, *HAL3* overexpression partially relieves loss of transcription of G1 cyclins in mutants lacking the protein phosphatase Sit4p, a protein required for passage from G1 to S phase in the cell cycle (Di Como *et al.*, 1995).

In this work we describe the isolation and posterior characterization of two *A. thaliana* genes coding for two proteins named AtHAL3a and AtHAL3b. The *Arabidopsis* proteins showed striking homology with yeast HAL3 and with a number of proteins from different organisms. Overexpression of *AtHAL3a* in the yeast *hal3* mutant partially complemented the salt sensitivity of the mutant. Recombinant AtHAL3a purified from *E. coli* was yellow and shown to be a flavin mononucleotide (FMN) flavoprotein. Finally, transgenic *Arabidopsis* plants overexpressing *AtHAL3a* showed altered growth phenotypes and improved salt and osmotic tolerance.

Results

Molecular cloning of AtHAL3a *and* AtHAL3b

An *Arabidopsis* cDNA (Stock 164P17T7) (R30079) identified in the course of an EXPRESSED SEQUENCE TAGS (ESTs) program (Newman *et al.*, 1994), has a deduced amino-acid sequence 52% identical to the yeast HAL3 protein. By using this cDNA as a probe, two genes with homology to yeast *HAL3* were isolated from an *A. thaliana* genomic library and named *AtHAL3a* and *AtHAL3b* (Figure 1a). The first gene corresponded to the cDNA 164P17T7. *AtHAL3a* and *AtHAL3b* genes contained, at the same position, an intron of 115 and 80 bp, respectively, and their open reading frames are 83% identical. Southern analysis of both genes (Figure 1b) demonstrates the expected cross-hybridization and also indicates that *Arabidopsis* does not contain other closely related genes. Despite the similarities within the

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*For correspondence (fax +34 6 3877859; e-mail faculia@ibmcp.upv.es).

open reading frames the similarity between the 5′ flanking regions was very low (41% identity), pointing to differences in expression of both genes.

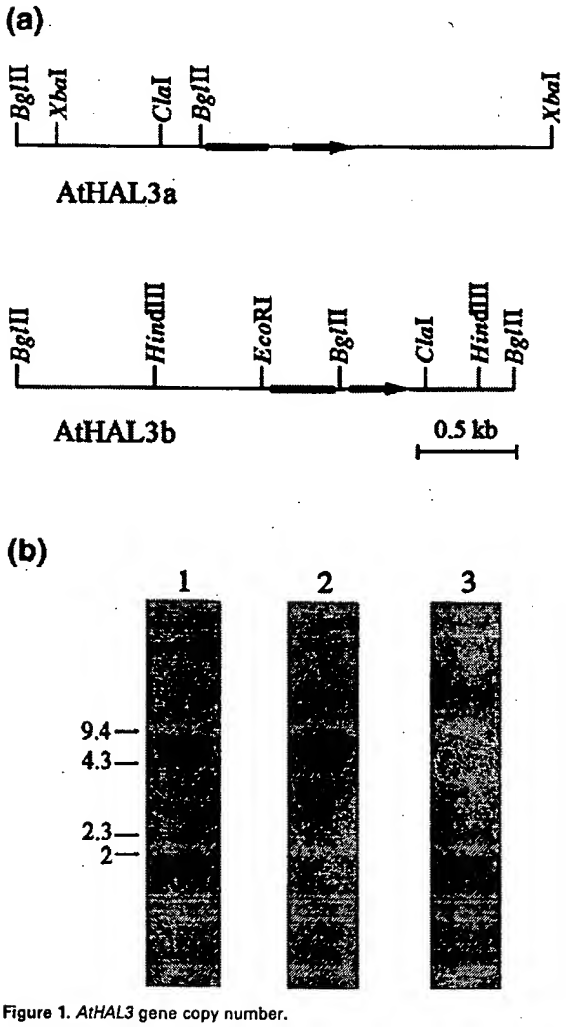


Figure 1. *AtHAL3* gene copy number.
(a) Restriction map of *AtHAL3a* and *AtHAL3b* genomic clones. Translated sequences are shown by thick arrows.
(b) Southern blot of *Arabidopsis* genomic DNA, digested with *Hin*dIII and hybridized with *AtHAL3a* cDNA (1), *AtHAL3a* 3′-untranslated (2) and *AtHAL3b* 3′-untranslated (3) radiolabelled probes at high stringency. Molecular markers are shown on the left in kDa.

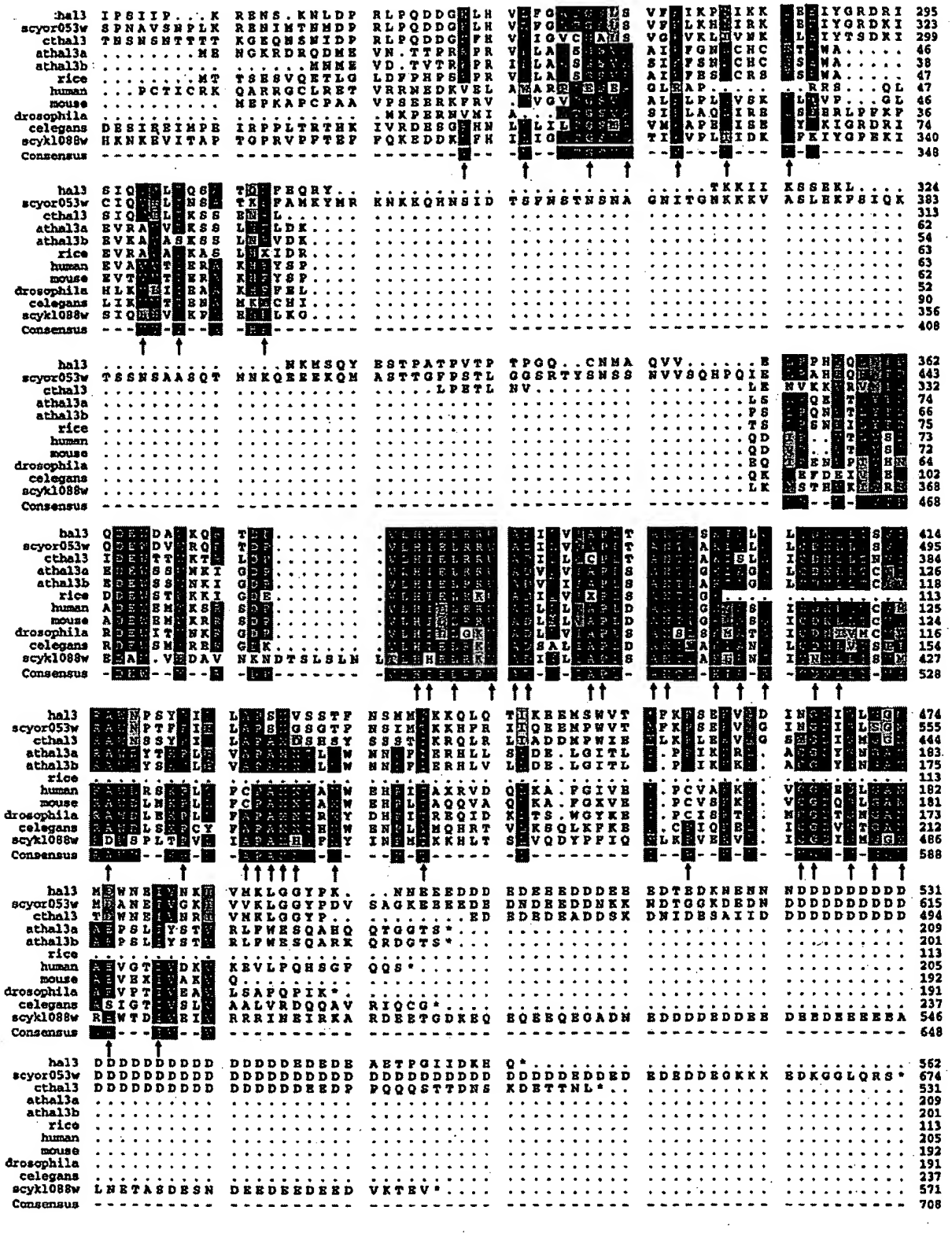
Identification of a novel homologous family

Comparison of the deduced *AtHAL3a* and *AtHAL3b* protein sequences with the Genebank database revealed a striking homology with other eukaryotic sequenced proteins from rice, humans, mouse, *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae* and *Candida tropicalis*. The regions of greatest similarity observed between the plant proteins are also conserved in the consensus derived from all family members (Figure 2). This homology occurs particularly in a central domain expanded along 180 amino-acid residues. Surprisingly, an aspartate- and glutamate-rich domain present in the carboxyl terminus of *C. tropicalis* and *S. cerevisiae* proteins is missing in the other family members. The rice C51401 protein is the most similar to AtHAL3a and AtHAL3b proteins (65–66% amino-acid identity). The plant proteins are more closely related to the animal than to the yeast homologous proteins. The eukaryotic family showed some homology with a large family of prokaryotic flavoproteins including, as key member, the *E. coli* DNA flavoprotein (DFP), involved in DNA replication (Spitzer and Weiss, 1985) and pantothenate metabolism (Spitzer *et al.*, 1988). The amino acids conserved between both families are indicated by arrows in Figure 2. Overall homology between the members of the eukaryotic family, however, is at least 33%, while the homology between eukaryotic and prokaryotic proteins of these families is less than 20%.

Expression of AtHAL3a *and* AtHAL3b

Figure 3 illustrates the expression of the two *Arabidopsis* genes in different tissues and under stress conditions. *AtHAL3a* and *AtHAL3b* expression was examined in roots, shoots, leaves, flowers, developing siliques and seeds (Figure 3a). Transcripts of *AtHAL3a* were detectable on Northern blots of poly(A)+ RNA from all these organs, particularly in flowers, siliques and seeds. The highest expression observed was in dry seeds, pointing to accumulation of *AtHAL3a* mRNA during *Arabidopsis* embryogenesis. The expression of *AtHAL3b* follows the pattern of *AtHAL3a* except in seeds, but the levels of transcript were considerably lower (particularly in seeds).

Figure 2. AtHAL3a and AtHAL3b alignment with homologous proteins.
Alignment of predicted amino-acid sequences for *Arabidopsis* AtHAL3a and AtHAL3b (accession numbers AF166262 and AF166263, respectively) to other related proteins from rice C51401 (C27242), human clone 730510 5′ (AA412663), mouse clone 1382198 5′ (AA798287), *Drosophila* CKO1102 3′ (AA141034), *Caenorhabditis elegans* cosmid (Z81069), yeast SIS2/HAL3 haloprotein (P36024) (Di Como *et al.*, 1995; Ferrando *et al.*, 1995), yeast open reading frame YOR053w (Z74961), *Candida tropicalis* CtHAL3 (X88900) (Rodríguez *et al.*, 1996), and yeast open reading frame YKL088w (Z28088), using the progressive alignment method of Feng *et al.* (1987). Residues are in dark boxes if six of 10 residues at a position are identical. Conserved residues are in grey boxes. The consensus sequence from all family members is shown below. Complete sequences show an asterisk at the end. The consensus homology with a large family of panthotenate metabolism flavoproteins (DFP), including *Escherichia coli* DFP (P24285) (Spitzer and Weiss, 1985), *Haemophilus influenzae* (P44953), *Synechocystis* sp. (D90910), *Bacillus subtilis* (Y13937), *Helicobacter pylori* (AE000595) and *Bradyrhizobium japonicum* (AF042096), is shown by arrows.


hal3
scyor053w
cthal3
athal3a
athal3b
rice
human
mouse
drosophila
celegans
scykl088w
Consensus

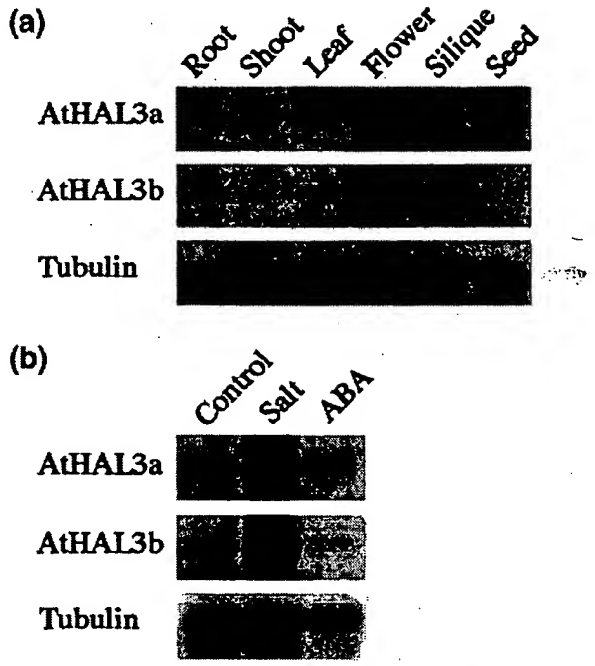


Figure 3. Expression of *AtHAL3a* and *AtHAL3b* mRNA during development and under stress treatments.
Northern blot of *Arabidopsis* poly(A)+ RNA probed with radiolabelled *AtHAL3a* and *AtHAL3b* cDNA.
(a) Different tissues.
(b) 12-day-old seedlings cultivated on MSS medium (control), MSS+100 mM NaCl (salt) and MSS+10 μM ABA.
The same filter was hybridized successively with *AtHAL3b* 3'-untranslated, *AtHAL3a* 3'-untranslated, and tubulin ^{32}P-labelled probes. Tubulin was used as loading control.

AtHAL3a and *AtHAL3b* expression was also examined in seedlings subjected to salt stress (100 mM NaCl) and ABA (10 μM) treatments (Figure 3b). ABA treatment did not show any effect on expression, while salt stress induced the expression of both genes. NaCl was a potent inducer of *AtHAL3b* expression, which under salt stress reached expression levels similar to those of *AtHAL3a*.

Spatial pattern of AtHAL3a *mRNA accumulation during* Arabidopsis *embryogenesis and in vegetative tissues*

Figure 4 shows the spatial and temporal pattern of *AtHAL3a* expression during embryo maturation and in vegetative tissues, further investigated by *in situ* hybridization of *AtHAL3a* mRNA. Fixed paraffin sections were hybridized with digoxigenin-labelled *AtHAL3a* antisense or sense strand probes to locate *AtHAL3a* mRNA sequences. During embryogenesis, *AtHAL3a* mRNA is detected in the cotyledons and hypocotyl of mature seeds; the *AtHAL3a* antisense probe produced intense hybridization, staining being mainly associated with embryo-specialized cell types from hypocotyl and cotyledon provascular tissues; a lower level of hybridization also occurs in the seed coat outer tegument and in the silique epidermis (Figure 4a,b). Accumulation of *AtHAL3a* mRNA was also observed in flower, shoot, leaf and root (Figure 4d–e, g–h, i–k, n–o, respectively), staining being restricted to differentiated cells from vascular elements of the vegetative tissues. In general, it seems that *AtHAL3a* expression is highly enriched in the phloem part of vascular tissue. *In situ* hybridization with a *AtHAL3a* mRNA control probe was used to monitor background hybridization. The specificity of the reaction was shown by the lack of appreciable reaction of the *AtHAL3a* sense-strand probe with the paraffin-embedded sections (Figure 4c,f,i,m,p).

Expression of AtHAL3a *in yeast* hal3 *mutants increases the tolerance to* Li^+

In order to test if *AtHAL3* is a functional homolog of yeast *HAL3*, we have expressed *AtHAL3a* in a yeast strain devoid of functional *HAL3*. As *hal3* mutants are sensitive to sodium and lithium (Ferrando *et al.*, 1995), we have tested if expression of *AtHAL3* complements these phenotypes. One striking difference between yeast HAL3 and *Arabidopsis* AtHAL3 is the presence in the fungal protein of a long acidic tail which has been reported to be essential to improve NaCl tolerance (Ferrando *et al.*, 1995) and to improve the growth of *sit4* mutants (Di Como *et al.*, 1995). Accordingly, we have included in our complementation studies both a truncated yeast HAL3, devoid of the acidic tail, and a chimeric AtHAL3 where the yeast acidic tail was fused to the AtHAL3 coding sequence. Figure 5 shows the lithium tolerance of yeast strain RS48 (*hal3* null mutant) transformed with different constructions including: yeast *HAL3*, yeast *HAL3* without the acidic tail, *AtHAL3a*, and a chimeric gene consisting of *AtHAL3a* fused with the yeast *HAL3* acidic tail. All the constructions were based in the high-copy number vector pRS699, which carries the constitutive strong PMA1 promoter. Cells transformed with empty vector were used as control. The effect of genetic dosage was studied using strain RS16 (wild type) which presents one copy of the wild-type *HAL3* gene. To confirm that all the different transformants grew equally well in the absence of salt stress, growth control tests were performed on YPD solid media (Figure 5a). In 100 mM LiCl (Figure 5b), AtHAL3a partially rescues the salt sensitivity exhibited by the *hal3* mutant; lithium tolerance slightly increases in cells expressing the chimera of *AtHAL3* fused to the yeast *HAL3* acidic tail. Complementation of lithium tolerance with a plasmid containing yeast *HAL3* showed little dependence on the presence of an acidic tail. Essentially identical results were obtained in drop tests with different LiCl concentrations (80, 150 and 200 mM LiCl; data not shown). Tolerance to 1 M NaCl, however, was more demanding on the type of HAL3 construct. AtHAL3a failed to complement the salt sensitivity of the *hal3*

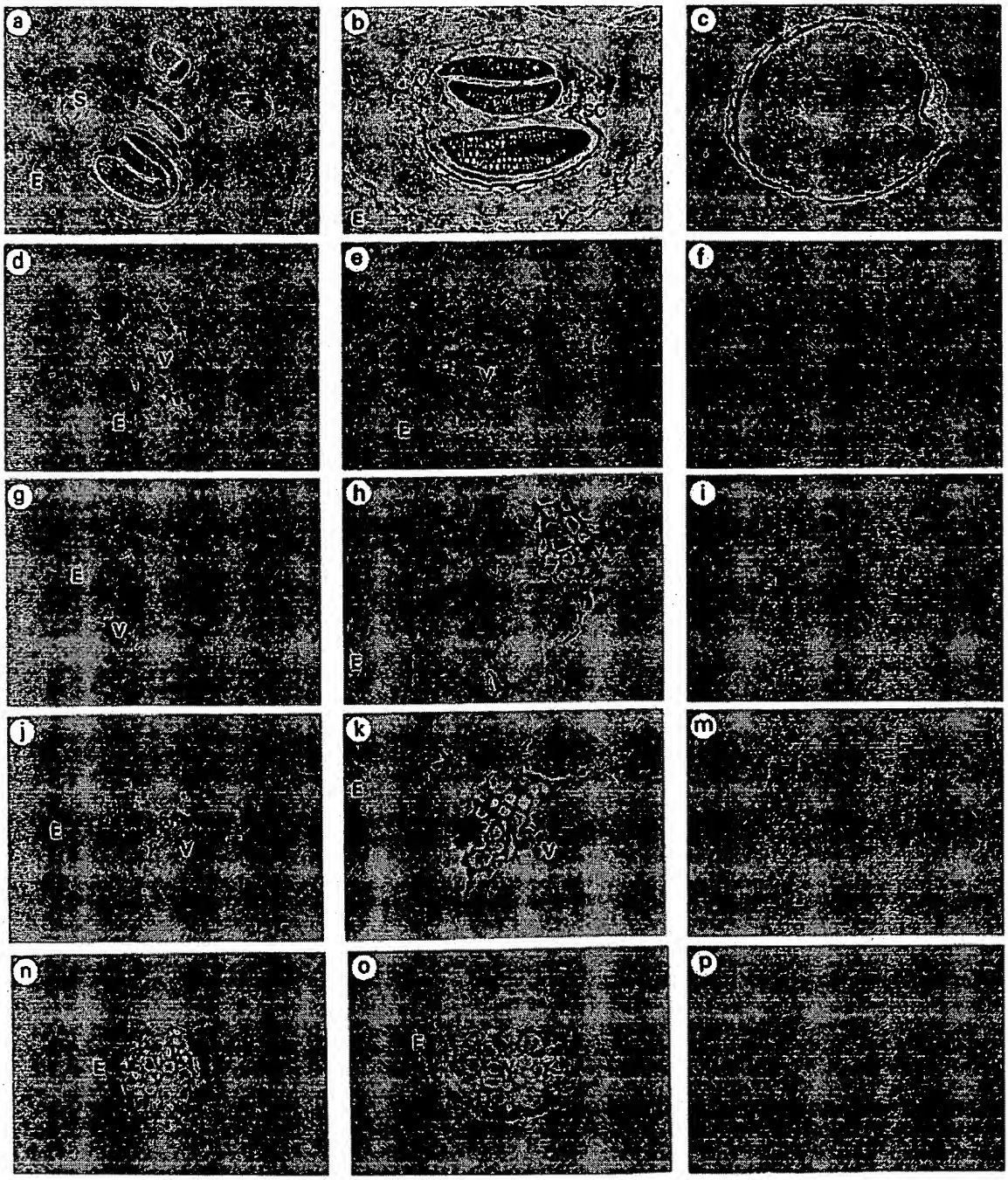


Figure 4. Localization of *AtHAL3* transcripts in seeds and different organs of *Arabidopsis*.
Paraffin-embedded sections hybridized with digoxygenin-labelled antisense *AtHAL3a* and viewed under bright field which gives a blue label.
(a,b) Transversal sections of mature silique; (d,e) longitudinal sections of flower; (g,h) transverse sections of shoot; (j,k) transverse sections of leaf; (n,o) transverse sections of root; (c,f,i,m,p) controls hybridized with *AtHAL3a* sense probe. Abbreviations: E, epidermis; S, seed; SC, seed coat; V, vascular tissue. Magnification ×50 in (a,d,g,j,n); ×100 in (b,c,e,f,h,i,k,m,o,p).

mutant, and the chimera with addition of the yeast acidic tail was also without effect. Essentially identical results were obtained in different NaCl concentrations (0.8 and 1.2 M NaCl, data not shown).

Identification of AtHAL3 as a flavoprotein

In order to characterize AtHAL3 protein, we expressed it in *E. coli* with a poly-histidine tail and purified it by nickel

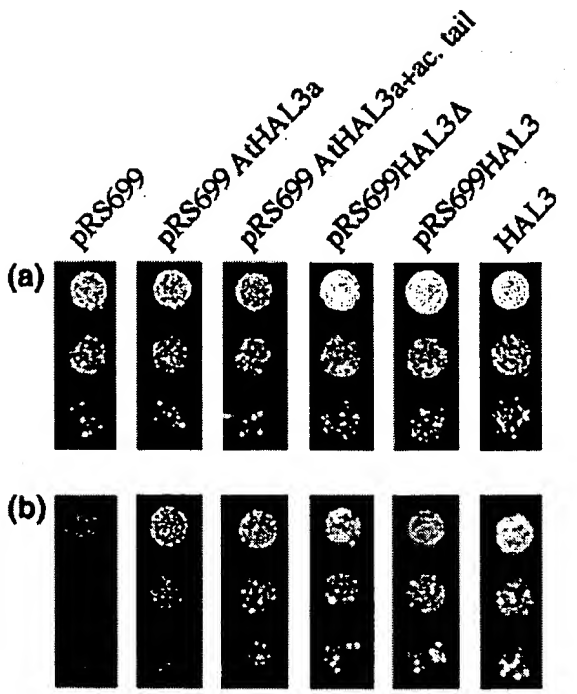


Figure 5. Complementation of the *S. cerevisiae hal3* null mutant with *AtHAL3a*.
(a) YPD solid media; (b) YPD+100 mM LiCl. Drops represent three different dilutions of saturated cultures, 1/10 (top), 1/100 (middle) and 1/1000 (bottom), incubated at 28°C for 36 h (a) and 7 days (b). *hal3* null mutant strain RS48 (RS16 *hal3::LEU2*) was transformed with the high-copy number vector pRS699 carrying different constructions: (pRS699) empty vector; (pRS699 AtHAL3a) *AtHAL3a*; (pRS699 AtHAL3a+ac.tail) *AtHAL3a* fused to *HAL3* acidic tail; (pRS699 HAL3Δ) truncated *HAL3* without acidic tail; (pRS699 HAL3) *HAL3*. (HAL3) wild-type strain RS16. Results were identical using three different transformants of each construct.

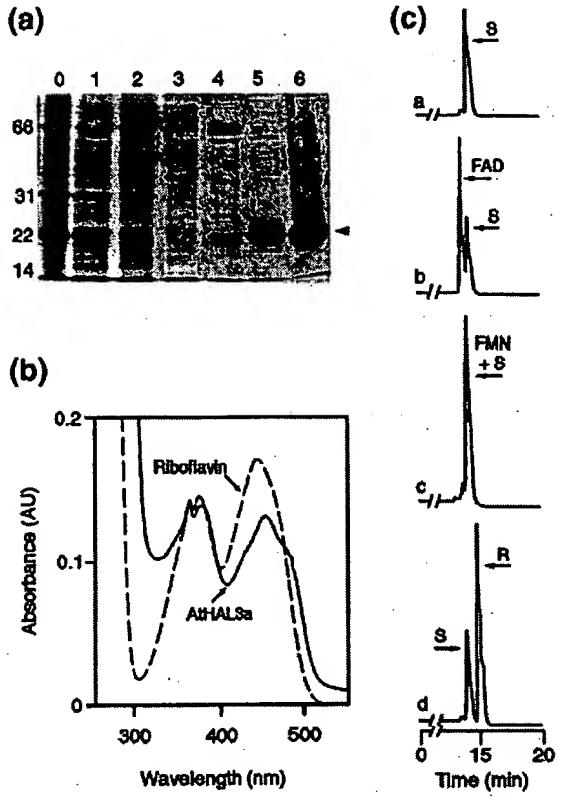


Figure 6. SDS-PAGE analysis of the *AtHAL3a* gene product and spectroscopic characterization of purified AtHAL3a.
(a) SDS-gel electrophoresis of His.Bind metal chelation resin (pET System, Novagen) fractions. The numbers above the tracks refer to the column fractions: (0) total protein extract from *E. coli* transformed with plasmid pET28-AtHAL3 (containing the coding region of the *AtHAL3a* cDNA), before column purification; (1–6) different column-eluted fractions after column purification. The 12% polyacrylamide gel was stained with Coomassie blue. Protein markers are shown on the left in kDa. AtHAL3a is indicated with an arrow.
(b) Absorbance spectrum of the 23 kDa AtHAL3a flavoprotein. The solid line is the spectrum of column fraction 6 from (a). The dashed line is the spectrum of 4 mM riboflavin. Samples were scanned in a Pharmacia 2000 spectrophotometer.
(c) Identification by HPLC of the fluorescent chromophore. 10 µl fraction 6 supernatant after acid treatment was either injected alone (a) or co-injected with 1 nmol of standard FAD (b), FMN (c) or riboflavin (d), respectively. Note the overlap between sample and standard FMN peaks in (c). Abbreviations: S, sample; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; R, riboflavin.

affinity chromatography. Figure 6 shows the SDS-gel electrophoresis of different protein fractions, the absorption spectra of the protein and the coenzyme identification. AtHAL3 was isolated from fraction 6, containing a partially purified protein, that migrated according to the predicted molecular mass (23 kDa) (Figure 6a). This band was absent in the control bacterial strain (data not shown). The most striking feature of the AtHAL3 preparation was its yellow color, pointing to the presence of a chromophore. Partially purified AtHAL3a showed the typical absorption spectrum of an oxidized flavoprotein (Williams, 1976; Kupke *et al.*, 1992), similar to the one showed by a characteristic flavin (riboflavin) (Spitzer and Weiss, 1985) (Figure 6b). The coenzyme released from the protein by heat precipitation, a treatment that should release noncovalently bound fluorescent chromophores (Koziol, 1971; Spitzer and Weiss, 1985), was identified by HPLC as FMN, as assessed by its co-elution with standard FMN (Figure 6c). The same results were obtained under different chromatographic conditions (data not shown). From the specific fluorescence of the preparation, the molar relationship of FMN and protein was 0.8, suggesting one molecule of FMN bound per molecule of AtHAL3a.

Sequence alignments for the cofactor-binding region of the flavin domain of key members of the FMN-containing family, bacterial flavodoxin (Watenpaugh *et al.*, 1973), yeast old yellow enzyme 12-oxophytodienoate reductase (OPDA reductase) (Saito *et al.*, 1991), bacterial oxidoreductase epidermin (EPID) (Kupke *et al.*, 1992), rat liver

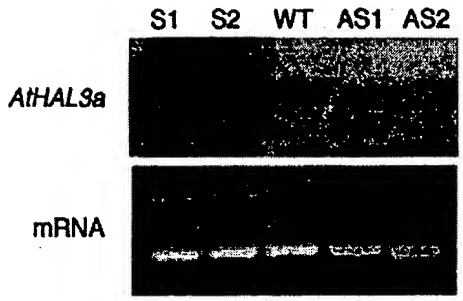


Figure 7. *AtHAL3a* constitutive expression on *Arabidopsis* transgenic lines.
Northern blot of *Arabidopsis* poly(A)+ RNA probed with radiolabelled *AtHAL3a* antisense riboprobe. S1 and S2, F_2 homozygous sense lines; WT, control wild type; AS1 and AS2, F_2 homozygous antisense lines.

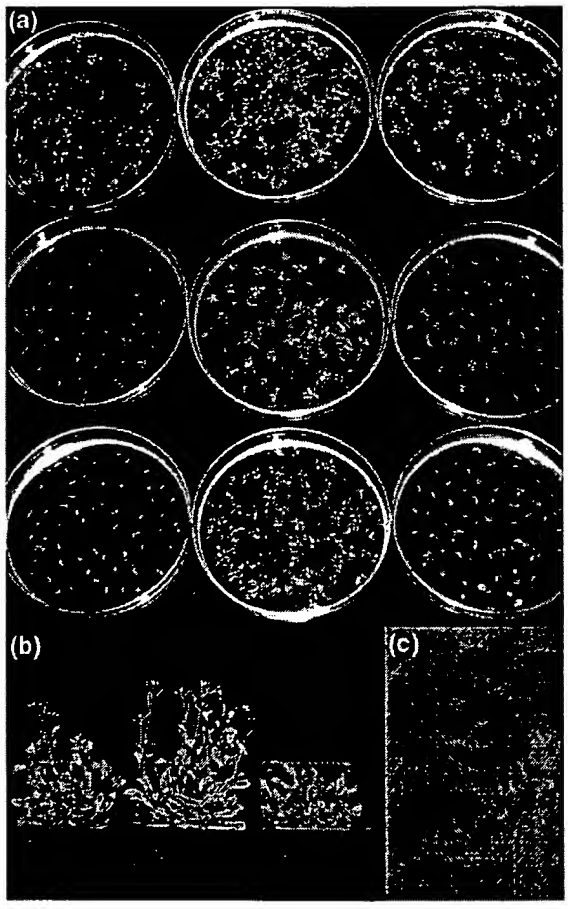


Figure 8. Effect of *AtHAL3a* constitutive expression on *Arabidopsis* stress tolerance and plant growth.
(a) *Arabidopsis* wild-type (left) and *AtHAL3a* transgenic F_2 homozygous lines sense S1 (centre) and antisense AS1 (right) on medium: MSS (top plates), MSS+100 mM NaCl (middle plates) and MSS+200 mM sorbitol (bottom plates), after 12 days' culture.
(b) *Arabidopsis* wild-type (left) and *AtHAL3a* transgenic F_2 homozygous lines sense S1 (centre) and antisense AS1 (right), after 40 days' culture.
(c) Closer view of plants of wild-type (left) and sense S1 (right) from (a) (middle plates).

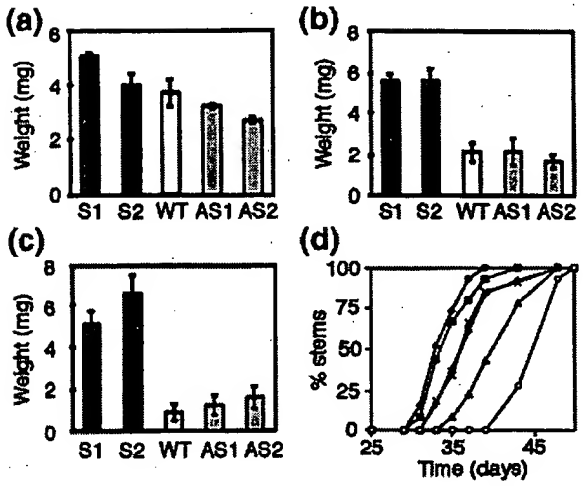


Figure 9. Growth comparison between *Arabidopsis* wild-type and *AtHAL3a* transgenic plants.
(a) Dry weight of plants on MSS media after 12 days' culture. S1 and S2, F_2 homozygous sense lines; WT, control wild type; AS1 and AS2, F_2 homozygous antisense lines. (b) As (a), but on MSS+100 mM NaCl. (c) As (a), but on MSS+200 mM sorbitol. (d) Comparative time course of stem production between F_2 homozygous sense lines S1 (◆) and S2 (■), control lines wild type (+) and F_2 homozygous transformed with the binary vector pBin19 (×), and F_2 homozygous antisense lines AS2 (Δ) and AS1 (O).
Each data point in (a–c) is the weight of 10 plants and corresponds to the mean value calculated from 50 plants of each line from three different experiments, and in (d) corresponds to the value, expressed in number of floral stems, calculated from 15 plants of each line. Error bars correspond to the standard error.

microsomal NADPH-cytochrome P_{450} reductase (CPR) (Wang *et al.*, 1997), along with AtHAL3a, do not show conserved polypeptide fragments in AtHAL3a encompassing the FMN binding site. However, the CPR structural X-ray determinations have showed that the FMN isoalloxazine ring-binding domain (five-stranded parallel β-sheets flanked by five α-helices) is approximately 170 amino acids long (Wang *et al.*, 1997), similar in length to the consensus domain shared by AtHAL3a and the prokaryote flavoprotein family (see arrows in Figure 2).

Transgenic Arabidopsis *overexpressing sense and anti-sense* AtHAL3a

Using *Agrobacterium*-mediated transfer, the *AtHAL3a* gene was stably integrated, in sense and antisense orientation, in the plant genome and expressed in the transgenic plants. F_1 plants were checked for transgene integration and *AtHAL3a* expression by PCR and Northern blot, respectively (not shown). Two sense (S1 and S2) and two antisense (AS1 and AS2) homozygous F_2 transgenic lines, that respectively showed increased and decreased *AtHAL3a* transcript amounts compared with the wild type, were chosen for further analysis (Figure 7).

Figures 8 and 9 show the effect of constitutive altered expression of *AtHAL3a* on *Arabidopsis* growth and stress tolerance. Plants that overexpress *AtHAL3a* gene show a faster growth rate than the wild type, while the *AtHAL3a* antisense plants produce the opposite phenotype (Figure 8a, top and Figure 9a). The differential growth rate observed is constant during plant development, particularly affecting the time course of floral stem production (Figures 8b and 9d). Sense plants also showed improved salt (Figure 8a, middle and Figure 9b) and osmotic (Figure 8a, bottom and Figure 9c) tolerance compared to wild-type and transgenic antisense plants. This improved tolerance is observed during germination and development. Sense transgenic plants developed roots and true leaves and continued growing under stress conditions, while wild-type plants mainly remained at the cotyledon stage (Figure 9c).

Discussion

In our search for plant genes homologous to the yeast halotolerance gene *HAL3*, we have identified a novel family of eukaryotic flavoproteins. Homology between yeast HAL3 and *Arabidopsis* AtHAL3a and AtHAL3b was restricted to a central domain of 180 amino acids, and database searches revealed a number of sequences from fungi, plants and animals which share the same domain. Its conservation through evolution points to a fundamental role in the physiology of eukaryotic cells. On the other hand, the acidic tail of fungal HAL3, mainly composed of glutamic and aspartic acid residues, is missing in the homologous proteins from plants and animals. The function of this acidic tail in the case of yeast HAL3 is not clear. It is important for sodium tolerance (Ferrando *et al.*, 1995) and to stimulate the growth of *sit4* mutants (Di Como *et al.*, 1995) but it is little required for lithium tolerance (present work). However, addition of the yeast acidic tail to AtHAL3a improves the complementation of yeast *hal3* mutants by the homologous plant protein. Yeast HAL3 is an inhibitory subunit of the protein phosphatase PPZ1 (Nadal *et al.*, 1998). It is plausible that the acidic tail participates in fostering the association of HAL3 with PPZ1, and that this reinforced association is required for some phenotypes of HAL3 but not for others, where a weaker interaction mediated by the conserved core could suffice. It would be interesting to determine whether plant and animal proteins homologous to yeast HAL3 interact with protein phosphatases related to yeast PPZ1.

In yeast, the protein phosphatase PPZ1 and its regulatory subunit HAL3 act as important determinants of salt tolerance by regulating the expression of the *ENA1* gene, encoding the major sodium extrusion pump of *S. cerevisiae* (Ferrando *et al.*, 1995; Nadal *et al.*, 1998). In addition, HAL3, probably via its interaction with PPZ1, also modulates the yeast cell cycle by regulating the expression of G1 cyclin genes (Di Como *et al.* 1995). A third function of the HAL3-PPZ1 regulatory complex is the modulation of the yeast MAP kinase pathway determining cell wall integrity (Nadal *et al.*, 1998). Therefore HAL3, probably by its interaction with the PPZ1 protein phosphatase, regulates genes concerned with ion homeostasis, cell cycle and cell wall integrity. Given the partial complementation of yeast *hal3* mutants by the *Arabidopsis* HAL3 homolog AtHAL3a, we speculate that plant (and probably animal) HAL3 homologous proteins may have similar mechanisms of action to the corresponding yeast gene. It could very well be that HAL3 proteins of plants and animals regulate, via a PPZ1-like protein phosphatase, the expression of genes related to the cell cycle, ion homeostasis and osmotic stability.

A bacterial family of flavoproteins also shows a significant homology to the conserved central domain of the eukaryotic homologous family. Interestingly, the homologous sheared region precisely expanded the eukaryotic conserved domain. These prokaryotic flavoproteins, like AtHAL3a, contain the flavin cofactor FMN. Because flavin coenzymes are involved in oxireduction reactions, an oxidoreductase activity has been suggested for these bacterial proteins, supposedly involved in an oxidative pathway of DNA synthesis and pantothenate metabolism (Spitzer and Weiss, 1985; Spitzer *et al.*, 1988). It would be interesting to investigate whether eukaryotic HAL3 homologous proteins experience redox changes, and if these changes influence their interaction with other proteins such as PPZ1.

Some clues for the physiological role of *Arabidopsis* HAL3 homologs may be provided by its pattern of expression. *AtHAL3a* is expressed at similar levels in most organs. The higher mRNA accumulation occurs in mature seeds. *AtHAL3b* follows the pattern of *AtHAL3a* expression, although its transcript levels were much lower (particularly in seeds). However, *AtHAL3b* was induced under salt stress to reach expression levels similar to *AtHAL3a*. The divergence in the 5′ upstream sequences between both genes probably reflects the differences in their expression.

In situ localization of mRNAs revealed that *AtHAL3a* expression was mainly restricted to the phloem part of vascular tissues. During embryogenesis, *AtHAL3a* mRNA is detected in the cotyledons and hypocotyl of mature seeds, mainly associated with embryo-specialized cell types from hypocotyl and cotyledon provascular tissues. This spatial pattern of expression during seed development is very similar to that shown by the late-embryogenesis-abundant genes maize *Rab28* (Niogret *et al.*, 1996), *Arabidopsis Atrab28* (Arenas-Mena *et al.*, 1999) and *Arabidopsis* peroxiredoxin antioxidant *AtPer1* (Haslekäs *et al.*, 1998). Since developing vascular centres play a major role in a variety of developmental processes (Nelson

and Langdale, 1992), and hormones and peptides are transported through the vascular system (Pearce *et al.*, 1991), this accumulation in cells of developing tissues and vascular structures is thought to be involved in late embryo-differentiation processes (Niogret *et al.*, 1996). AtHAL3a may well be involved in the cell cycle of developing vascular tissues, while in mature phloem it could be part of a signal-transduction pathway for defence against osmotic stress.

Transgenic *Arabidopsis* plants constitutively over-expressing *AtHAL3a* showed improved growth and salt and osmotic tolerance. The antisense plants, despite their altered growth phenotype, do not show any stress-hypersensitive response. These phenotypes could well be a consequence of the strength of AtHAL3a control on the expression of genes related to the cell cycle, ion homeostasis and osmotic stability during embryogenesis and in the phloem of vegetative tissues. Indeed, the fact that gain and loss of AtHAL3a activity correlate with the growth rate of *Arabidopsis* plants points to an important rate-limiting role in developmental growth.

It is expected that these novel flavoproteins will be implicated in many plant processes. Biochemical and X-ray analysis are in progress to unravel the possible redox activity and the structure of AtHAL3a, in order to gain more insight into the mechanisms of action of these new plant flavoproteins.

Experimental procedures

Plant material and stress treatments

Arabidopsis thaliana ecotype Columbia was grown in the greenhouse at 25°C under 8 h dark, 16 h light. For seedling stress, wild-type and transgenic surface-sterilized *Arabidopsis* seeds were sown in Petri dishes containing 25 ml MSS medium [MS (Murashige and Skoog, 1962) + 3% sucrose], MSS+100 mM NaCl for salt stress, MSS+200 mM sorbitol for osmotic stress, and MSS medium supplemented with 10 μM ABA (48 h after day 12) for ABA treatments. Seedlings were grown for 12 days at 25°C under fluorescent light, 8 h dark and 16 h light.

Isolation of genomic clones

Approximately 50 000 plaque-forming units, from an EMBL3 library of *A. thaliana* DNA partially digested with *Sau*3A (Clontech, Palo Alto, CA, USA), were screened with the *AtHAL3a* cDNA (Stock 164P17T7) obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, OH, USA). After washing at low stringency (55°C in 2 × SSC, 0.5% SDS), five positive clones were isolated that hybridized with *AtHAL3a*. The genomic clones were mapped with different restriction enzymes and were shown to be not identical. Three of these clones contained *AtHAL3a* and the other two contained *AtHAL3b*. Genomic clones AG1 and AG5, which contain *AtHAL3a* and *AtHAL3b* coding regions, respectively, were selected for further analysis.

Isolation of cDNA clones

300 000 plaque-forming units from an *A. thaliana* Uni-ZAP XR library (Stratagene, La Jolla, CA, USA), constructed from 4-week-old adult plants grown under long-day conditions, were also screened with the *AtHAL3a* cDNA (Stock 164P17T7). After washing at low stringency (2 × SSC, 0.5% SDS, at 55°C), nine positive cDNA clones were obtained, all of which contained *AtHAL3a*. cDNA clone AC8, with the complete *AtHAL3a* coding sequence, was selected for subsequent experiments. AC8 cDNA sequences can be deduced joining nucleotides 888–1289 and 1407–1867 from accession number AF166262.

Plasmids, yeast strains and culture conditions

Yeast strains used in this study were RS16 (wild type) (Gaxiola *et al.*, 1992) and RS48 (RS16 *hal3::LEU2*) (Ferrando *et al.*, 1995). Transformants were obtained using the lithium acetate procedure (Ito *et al.*, 1983). Cells were routinely grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose). Transformants were selected by plating on minimal medium (SD) containing 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco) and 50 mM MES adjusted to pH 5.5 with Tris. Salt tolerance was determined in solid YPD medium (containing 2% bacteriological grade agar) supplemented with NaCl and LiCl to the indicated final concentrations by a drop assay. The *AtHAL3a* coding sequence was PCR amplified from AC8 adding extra 5'-end *Hin*dIII and 3'-end *Bam*HI restriction sites and cloned *Hin*dIII/*Bam*HI into pBluescript SK+ (Stratagene) creating plasmid pA8. Then it was cloned as a 630 bp *Xho*I fragment, PCR amplified from pA8 creating extra *Xho*I restriction sites at both ends, in the appropriated orientation, into yeast vector pRS699 (Serrano and Villalba, 1995) which contains the strong constitutive *PMA1* promoter, creating pRS699 *AtHAL3a*. The *HAL3* coding region was introduced into pRS699, as described above for *AtHAL3a*, as a 1.7 kb *Xho*I fragment from plasmid pA44 (*HAL3* coding region, cloned *Eco*RI/*Bam*HI in vector pUC19, New England Biolabs), to produce pRS699 *HAL3*. The 1.4 kb *Eco*RI/*Kpn*I fragment from pA44, which contains *HAL3* coding region lacking the acidic tail, was cloned into yeast vector pYES 2.0 (Invitrogen, CA, USA), creating pA308. The 1.4 kb *Xba*I/*Hin*dIII fragment from pA308, which adds an extra *Xho*I site at the 5' end, was subcloned into vector pBluescript SK(+) (Stratagene), creating pA321. The 1.4 kb *Xho*I fragment from pA321 was then cloned, in the appropriated orientation, into yeast vector pRS699 to produce pRS699 *HAL3Δ*. A chimeric gene containing *AtHAL3a* coding region plus *HAL3* acidic tail was constructed appending the 0.63 kb *Hin*dIII/*Kpn*I fragment from plasmid pA8 (containing *AtHAL3a* coding sequence) and the 0.24 kb *Kpn*I/*Pst*I fragment (containing *HAL3* acidic tail) isolated from plasmid pA44 and then cloned into *Hin*dIII/*Pst*I vector pBluescript SK(+), creating pA348. The chimera was finally isolated from pA348 by PCR adding extra *Xho*I sites at both ends and cloned as a 0.9 kb *Xho*I fragment, in the appropriate orientation, into yeast vector pRS699 to produce pRS699 *AtHAL3*+ac tail.

Sequencing

PCR amplified DNA was sequenced for detection of possible mistakes. Sequence on both strands was determined according to Sanger *et al.* (1977) by double-stranded plasmid sequencing in pBluescript using Sequenase (United States Biochemicals). Sequence analysis was performed using the WISCONSIN Package

version 9.0 (Genetics Computer Group, Madison, Wisconsin, USA). Genomic *AtHAL3a and AtHAL3b* nucleotide sequences have been submitted to the Genebank/EMBL Databank under the accession numbers AF166262 and AF166263, respectively.

DNA gel blotting, Northern blots and hybridization

Genomic DNA gel blots and Northern analysis were performed using approximately 10 µg DNA and 5 µg poly(A)+ RNA per track, respectively. Isolated DNA fragments were nick-translated in the presence of α-[^{32}P]dCTP to be used as probes (Maniatis *et al.*, 1982). α-[^{32}P]CTP radiolabelled RNA probes were performed according to the manufacturer's instructions (Boehringer Mannheim). Probe *AtHAL3a* cDNA, was a 0.6 kb *Hin*dIII/*Bam*HI fragment from plasmid pA8; probes *AtHAL3a* and *AtHAL3b* 3'-untranslated, were two 180 and 200 bp *Hin*dIII/*Bam*HI fragments obtained by PCR amplification from genomic clones AG1 and AG5 3'-untranslated region, and to which extra 5'-end *Hin*dIII and 3'-end *Bam*HI restriction sites were added, respectively. *AtHAL3a* riboprobe was transcribed from linear *Hin*dIII pA8 using T3 RNA polymerase. Hybridization was performed in PSE (0.3 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA) at 65°C for Southern and at 55°C for Northern and in PSE+50% formamide at 55°C when RNA probe was used. Filters were washed at high stringency (0.1 × SSC, 0.5% SDS at 65°C).

In situ *hybridization*

For *in situ* hybridization, digoxigenin-labelled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim) and performed as previously described (Goday *et al.*, 1994). Sense and antisense probes were transcribed from *Bam*HI and *Hin*dIII linear pA8 using T7 and T3 RNA polymerases, respectively. In all cases, no signal over background was observed using control sense strand probes.

Chimeric AtHAL3a protein synthesis and purification

The full length of *AtHAL3a* coding region was PCR amplified from cDNA clone AC8 adding extra 5'-end *Bam*HI and 3'-end *Hin*dIII restriction sites and subcloned *Bam*HI/*Hin*dIII into vector pET-28a(+) (Novagen, MA, USA), overexpressed in *E. coli* and purified by affinity chromatography with His.Bind metal chelation resin (pET System, Novagen). Total protein yield was 5 mg. Protein extraction and electrophoresis were performed as previously described (Niogret *et al.*, 1996).

Preparation of extracts and HPLC analysis

To release the fluorescent chromophore, 0.5 ml from the purified enzyme fraction 6 were treated with perchloric acid (5% final concentration) at 0°C for 15 min. Protein was clarified by centrifugation at 2000 r.p.m. (1000 *g*) for 5 min and further treated as previously described (Murguía *et al.*, 1995, 1996). 10 ml of extract were analysed by HPLC in a Waters 600 E liquid chromatograph. Samples were injected onto a reversed-phase C18 column (Nova-Pak, 4 × 250 mm, 4 µm particle size, Waters) maintained at 25°C and equilibrated in 4% MeOH and 83.3 mM triethylammonium phosphate (pH 6.0) according to Lim (1991). After injection, a gradient of MeOH (4–100%), with a flow rate of 1 ml min^{-1}, was applied over 20 min. FAD, FMN and riboflavin were detected in a Waters 486 absorbance detector as described by Lim (1991). Peaks were identified by co-injection with standard. Peak areas were quantified with a Waters 746 integrator by comparison with known amounts of FAD, FMN and riboflavin standards.

Plant transformation

The *AtHAL3a* coding region was isolated by PCR from plasmid pA8, and cloned in sense and antisense orientation as a 0.63 kb *Hin*dIII/*Bam*HI fragment into plasmid pJIT 163 (Guerineau *et al.*, 1992), creating pA18 and pA61, respectively. The 2.1 kb *Kpn*I/*Xho*I DNA fragment from pA18 and pA61, which contains the *AtHAL3a* gene flanked by a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer and by the CaMV polyadenylation sequence, was finally cloned into the binary plant vector pBin19 (Bevan, 1984) creating pA31 and pA76, respectively. *Agrobacterium* helper strain LBA 4404 (Hoekema *et al.*, 1983), was transformed with pA31 or pA76 by high-voltage electroporation (Wen-Jun and Forde, 1989), and used for plant transformation. *Arabidopsis thaliana* adult plants (5 weeks old) were agroinfected by infiltration (Bechtold *et al.*, 1993) and grown in the greenhouse to collect seeds. F_0 seeds were grown in MSS medium supplemented with 50 mg ml^{-1} kanamycin (K4378, Sigma Chemical Company, MO, USA). A yield of one transformant per 1000 seeds was obtained. Ten independent kanamycin resistant F_0 plants were selected for each transformation, transferred to soil after 15 days and grown in greenhouse to collect seeds. F_1 plants were checked for transgene integration and *AtHAL3a* expression by PCR and Northern blot, respectively. Two lines for each transformation were selected which showed higher and lower expression of *AtHAL3a*, respectively, and which segregated 3 : 1 in kanamycin, as expected for a single integration of the construction in the plant genome. From each of the selected lines, 10 F_1 plants were grown and seeds were collected. F_2 plants were segregated in MSS media supplemented with 50 mg ml^{-1} kanamycin. One homozygous line was selected for each sense and anti-sense F_2, named S1, S2 and AS1, AS2, respectively, and used for phenotype characterization and stress treatments.

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References

Arenas-Mena, C., Raynal, M., Borrell, A., Varoquaux, F., Cutanda, M.C., Stacy, R.A.P., Pagès, M., Delseny, M. and Culiáñez-Macià, F.A. (1999) Expression and cellular localisation of *Atrab28* during *Arabidopsis* embryogenesis. *Plant Mol. Biol.* **40**, 355–363.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci.* **316**, 1194–1199.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plants transformation. *Nucl. Acids Res.* **12**, 8711–8721.

Boyer, J.S. (1982) Productivity and environment. *Science*, **218**, 443–448.

Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.* **2**, 48–54.

Di Como, C.J., Bose, R. and Arndt, T. (1995) Overexpression of *SIS2* which contains an extremely acidic region, increases expression of *SWI4*, *CLN1* and *CLN2* in *sit4* mutans. *Genetics*, **139**, 95–107.

Feng, D.F., Johnson, M.S. and Doolittle, R.F. (1987) Aligning amino acids sequences: comparison of commonly used methods. *J. Mol. Evol.* **21**, 112–125.

Ferrando, A., Kron, S.J., Rios, G., Fink, G.R. and Serrano, R. (1995) Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene *Hal3*. *Mol. Cell. Biol.* **15**, 5470–5481.

Gaxiola, R., Larrinoa, I.F., Villalba, J.M. and Serrano, R. (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**, 3157–3164.

Goday, A., Jensen, A., Culiáñez-Macià, F.A., Albà, M.M., Figueras, M., Serratosa, J., Torrent, M. and Pagès, M. (1994) The maize abscisic acid responsive protein Rab17 is located in the nucleus and cytoplasm and interacts with nuclear localisation signals. *Plant Cell*, **6**, 351–360.

Guerineau, F., Lucy, A. and Mullineaux, P. (1992) Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. *Plant Mol. Biol.* **18**, 815–818.

Haslekās, C., Stacy, R.A.P., Nygaard, V., Culiáñez-Macià, F.A. and Aelen, R.B. (1998) The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in *Arabidopsis thaliana* is seed specific and related to dormancy. *Plant Mol. Biol.* **36**, 833–845.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti plasmid. *Nature*, **303**, 179–180.

Holmberg, N. and Bülow, L. (1998) Improving stress tolerance in plants by gene transfer. *Trends Plant Sci.* **3**, 61–66.

Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alcali cations. *J. Bacteriol.* **153**, 163–168.

Koziol, J. (1971) Fluorometric analyses of riboflavin and its coenzymes. *Meth Enzymol.* **18A**, 253–285.

Kupke, T., Stevanovic, S., Sahl, H.-G. and Götz, F. (1992) Purification and characterization of EpiD, a flavoprotein involved in the biosynthesis of the lantibiotic epidermin. *J. Bacteriol.* **174**, 5354–5361.

Lim, C.K. (1991) Nucleotides. In *Liquid Chromatography in Biomedical Analysis* (Hanai, T., ed.), Journal of Chromatography Library Vol. 50. Amsterdam: Elsevier Science, pp. 191–208.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Murguía, J.M., Bellés, J.M. and Serrano, R. (1995) A salt sensitive 3′(2′), 5′-bisphosphate nucleotidase involved in sulfate activation. *Science*, **267**, 232–234.

Murguía, J.M., Bellés, J.M. and Serrano, R. (1996) The yeast *HAL2* nucleotidase is an *in vivo* target of salt toxicity. *J. Biol. Chem.* **271**, 29029–29033.

Nadal, E., Clotet, J., Posas, F., Serrano, R., Gómez, N. and Ariño, J. (1998) Regulation of salt tolerance by HAL3p is mediated through the PPZ1 Ser/Thr protein phosphatase. *Proc. Natl Acad. Sci. USA*, **95**, 7357–7362.

Nelson, T. and Langdale, J.A. (1992) Developmental genetics of C_4 photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 25–47.

Newman, T., de Bruijn, F.J., Green, P. *et al.* (1994) Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* **106**, 1241–1255.

Niogret, M.F., Culiáñez-Macià, F.A., Goday, A., Albà, M.M. and Pagès, M. (1996) Expression and cellular localization of *rab28* mRNA and Rab28 protein during maize embryogenesis. *Plant J.* **9**, 549–557.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, **253**, 895–899.

Rodríguez, P.L., Ali, R. and Serrano, R. (1996) CtCdc55p and CtHal3p: two putative regulatory proteins from *Candida tropicalis* with long acidic domains. *Yeast*, **12**, 1321–1329.

Saito, K., Thiele, D.J., Davio, M., Lockridge, O. and Massey, V. (1991) The cloning and expression of a gene encoding Old Yellow Enzyme from *Saccharomyces carlsbergensis*. *J. Biol. Chem.* **266**, 20720–20724.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-teminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Serrano, R. (1994) Yeast halotolerance genes: crucial ion transport and metabolic reactions in salt tolerance. In *Biochemical and Cellular Mechanisms of Stress Tolerance in Plants* (Cherry, J.H., ed.), NATO ASI Series, Vol. H86. Berlin: Springer-Verlag, pp. 371–380.

Serrano, R. and Villalba, J.M. (1995) Expression and localisation of plant membrane proteins in *Saccharomyces*. *Methods Cell. Biol.* **50**, 481–496.

Spitzer, E.D. and Weiss, B. (1985) *dfp* gene of *Escherichia coli* K-12, a locus affecting DNA synthesis, codes for a flavoprotein. *J. Bacteriol.* **164**, 994–1003.

Spitzer, E.D., Jiménez-Billini, H.E. and Weiss, B. (1988) β-Alanine auxotrophy associated with *dfp*, a locus affecting DNA synthesis in *Escherichia coli*. *J. Bacteriol.* **170**, 872–876.

Wang, M., Roberts, D.L., Paschke, R., Shea, T.M., Masters, B.S.S. and Kim, J.-J.P. (1997) Three-dimensional structure of NADPH-cytochrome P_{450} reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl Acad. Sci. USA*, **94**, 8411–8416.

Watenpaugh, D.K., Siekel, L.C. and Jensen, L.H. (1973) The binding of riboflavin-5′-phosphate in a flavoprotein: flavodoxin at 2.0-A resolution. *Proc. Natl Acad. Sci. USA*, **70**, 3857–3860.

Wen-Jun, S. and Forde, B.G. (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucl. Acids Res.* **17**, 8385–8385.

Williams, C.H. (1976). Flavin-containing dehydrogenases. In *The Enzymes* (Boyer, P.D., ed.). New York: Academic Press, pp. 89–173.

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